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Insertion of a rabbit β-globin gene sequence into an E.coli plasmid.

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ABSTRACT

Double stranded DNA has been synthesized in vitro from rabbit globin messenger RNA and elongated with homopolymeric dG tails. An E.coli plasmid was cleaved by EcoRI. The cohesive ends were repaired and dC tails added, to permit reconstitution of the EcoRI sites upon annealing with the dG elongated globin DNA. Transformation of E.coli with the globin-plasmid DNA hybrid has yielded a clone which harbours a recombinant plasmid (pCR1-βG1), as demonstrated by hybridization experiments with radioactive globin cDNA. The sequence carried by the recombinant plasmid corresponds to part of the gene sequence coding for the β chain of rabbit globin. Circular DNA of the purified recombinant plasmid exhibits sensitivity to EcoRI.

INTRODUCTION

Two possible strategies can be envisaged for the insertion of specific eukaryotic sequences into bacterial or viral DNA :

(1) Fragments of cellular DNA containing whole or part of a given gene can be inserted into a molecular vehicule such as a plasmid or a phage. This manipulation can be accomplished either by pairing the cohesive ends created by certain restriction endonucleases (1-2) or by hybridizing complementary homopolymeric tails synthesized enzymatically (3-4). Random insertion of DNA fragments can be followed by selection or screening for a given sequence among the cloned recombinants. Alternatively, the isolation of a given DNA fragment, or an enrichment, can preceed insertion.

(2) The second possibility is to use a given RNA sequence (cellular or viral) as a template for the in vitro synthesis of a DNA sequence which can then be integrated into a molecular vehicle for the purpose of cloning and amplification.

We have used double stranded DNA (ds-DNA) synthesized in vitro from a rabbit globin mRNA template for insertion into a bacterial plasmid. In this

report, we describe a new E.coli plasmid which carries a sequence specific of the rabbit gene coding for the β chain of globin.

MATERIALS AND METHODS

Isolation of plasmid DNA. E. coli plasmid pCRI was constructed by Dr. J. Carbon from plasmid pML2, which is the colE1 plasmid carrying the kanamycine resistant marker (5). As pML2, pCRI can be amplified by the addition of chloramphenicol,but pCRI has only one site of EcoRI action, while pML2 has 2. It was kindly provided by Dr. J.D. Rochaix. E. coli K12 harbouring pCRI was grown in mineral medium supplemented with glucose and casaminoacids (5). When the density reached 3 x 10^8 cells per ml, chloramphenicol was added at a final concentration of 200 µg/ml, and incubation was continued overnight (5). Cells were then centrifuged and the pellets were eventually stored at -30°C. Closed circular plasmid DNA was isolated by 2 cycles of density centrifugation in CsCl-Ethidium bromide gradients (6) followed by centrifugation in a neutral sucrose gradient (5 to 20 % sucrose in 10 mM Tris-Hcl pH 7.6, 2 mM EDTA,0.1 M NaCl, 4 hours at 37 000 rpm in the SW 41 rotor of a Spinco ultracentrifuge).

Purification of mRNAs. Rabbit 9 S globin mRNA was prepared as described (7). It was further fractionated into α and β globin mRNA by electrophoresis in 4.5 % acrylamide gels with 98 % formamide (8-9). The RNAs were then eluted and rechromatographed on oligodT-cellulose columns as described elsewhere (10). Cross-contamination is estimated at 10-20%.

Mouse immunoglobulin 14 S L chain mRNA was prepared from MOPC 41 myeloma tumors as previously described (11) with an additional purification in acrylamide gels (10). The synthesis of MOPC 41 L chain cDNA has also been described (12).

Enzymes. Avian myeloblastosis virus (AMV) DNA polymerase was a gift of Dr. J. Beard. Calf thymus deoxynucleotidyl terminal transferase (TdT) was purified to homogeneity according to Chang and Bollum (13). Single-strand specific nuclease from Aspergillus oryzae (S 1 nuclease) was purified according to the procedure of Vogt up to the DEAE cellulose step (14). Eco RI endonuclease was prepared according to Greene et al (15).E. coli DNA polymerase I (enzyme A according to Klenow et al (16)) was obtained from Boehringer (Mannheim).

Synthesis of complementary DNA. The reaction mixture contained : 50 mM Tris-HCl pH 8.2, 50 mM KCl, 7.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM (each) dATP, dTTP and dGTP, and (^{3}H)-dCTP (20 Ci/mMole), 50 µg/ml of actinomycin D, 10-50 µg/ml of either 9 S or 14 S mRNA, 1-5 µg/ml of dT_{12-18} (P-L Biochemicals) and 3 units of AMV DNA polymerase per µg of template. The incubation was for

1 hour at 37°C. After alkaline hydrolysis in 0.3 M NaOH the mixture was filtered on a G 75 Sephadex column (0.5 x 10 cm), in H_2O. The excluded material was precipitated with ethanol and fractionated in an alkaline sucrose gradient (12).

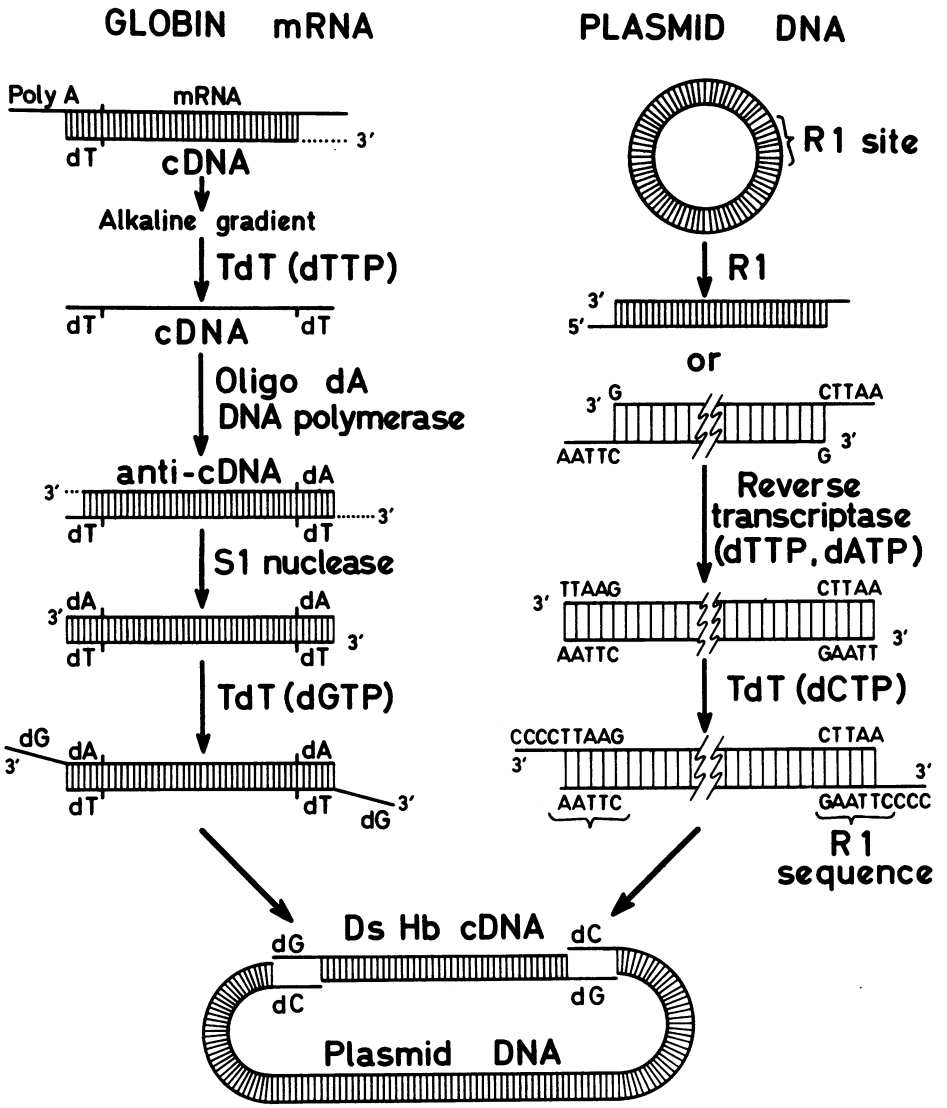

Figure 1. Schematic representation of the approach followed for the annealing of globin DNA to plasmid DNA.

Synthesis of the double stranded DNA. The details of the procedure will be published elsewhere (17). Briefly, unlabelled single-stranded cDNA with a minimum chain length of 200 nucleotides was isolated in a neutral sucrose gradient. About 20-40 dTMP residues on the average were added per molecule by terminal transferase. The dT elongated cDNA was annealed with oligodA_{10} and converted into a duplex structure in a mixture containing 50 mM Tris pH 7.6, 10 mM MgCl2, 20 mM KCl, 1 mM DTT, 0.2 mM (each) dATP, dTTP, dCTP, and dGTP and E. coli DNA polymerase. Incubation was for 30 min at 30 °C. After phenol extraction, the mononucleotides were removed by filtration on a G 75 Sephadex column in H_2O . Control experiments have shown that this procedure results in a well matched, globin mRNA-specific double stranded DNA. It was also observed that the conversion of cDNA into dsDNA is not entirely primer dependent. Both E.coli and AMV DNA polymerases are able to convert cDNA into dsDNA (17).

Elongation of ds globin DNA. Double stranded (ds) globin DNA was incubated for 30 minutes at 45°C with nuclease S 1 in a 0.1 ml mixture containing 0.1 M sodium acetate buffer pH 4.5, 1 mM ZnCl2, 0.2 M NaCl, 0.5 µg of ds-DNA. There was enough S 1 nuclease to digest 1 µg of denatured DNA in 60 minutes at 45°C. The mixture was extracted by chloroform-isoamylalcohol (24:1) and passed through a G 75 Sephadex column in H_2O. Homopolymeric extension of the duplex was achieved by incubation of 0.5 µg of ds- DNA in a 0.05 ml mixture containing : 0.1 M Hepes pH 7.2, 8 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.2 mM dGTP, and terminal transferase for 1 hour at 37 °C. This resulted in the addition of about 10 residues of dGMP per 3' OH terminus on the average. After S 1 digestion of the ds-DNA, the length of the anti-cDNA strand, determined by electrophoresis in acrylamide gels in 98 % formamide with appropriate molecular weight markers, was heterogeneous, ranging from 100 to 300 nucleotides (17).

Addition of homopolymer to linear pCR1 plasmid DNA molecule. Closed circular pCR1 DNA was converted into linear molecules by incubation in a mixture containing 100 µg per ml of DNA, 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$ and Eco RI endonuclease. After 1 hour at 37°C the mixture was made 50 mM in EDTA and extracted with phenol. Linear molecules were isolated by sedimentation at 20 ° in a neutral 5-20 % sucrose gradient (10 mM Tris HCl pH 7.6, 0.1 M NaCl, 1 mM EDTA) for 4 h. at 37.000 rpm in a SW 41 rotor. After ethanol precipitation of the DNA, the 3'OH termini generated by EcoRI were repaired as described (18), using AMV DNA polymerase as the repair enzyme. The incorporation was that expected from complete repair. The mixture was phenol extracted and filtered through a Sephadex G 75 column and the excluded fractions were precipitated with ethanol. The repaired pCR1 (20 µg/ml) in 0.1 M Hepes (pH 7.2), 8 mM

$MgCl_2$, 1 mM β-mercaptoethanol, 0.2 mM dCTP was incubated in the presence of terminal transferase at 37°C in siliconized glass tubes. The addition of dCMP residues was determined by the measure of the (^{3}H) dCMP incorporated in TCA precipitable material. When 20 monomers per terminus on the average were added, the mixture was phenol extracted and precipitated with ethanol. Control experiments showed that intact circular molecules did not act as primers for the terminal transferase. This suggests that the enzyme does not introduce a significant number of nicks in the duplex plasmid DNA.

Annealing of the pCR1 DNA with globin ds- DNA. The polydC- elongated pCR1 DNA (1 µg/ml) and the poly dG-elongated globin ds-DNA (about 0.01 µg/ml) were annealed in Tris 10 mM (pH 7.6) 0.1 M NaCl, 0.1 mM EDTA for 4 hours at 65°C. Then the mixture was allowed to cool slowly (in about 4 hours) at room temperature.

Transformation. Transformation of C600 (r_k^- m_k^- $recBC^-$) was carried out as described (2). Before plating, the cells (0.3 ml) were incubated with L broth (0.3 ml) for 45 min at 37°C. The plates contained 20 µg/ml of kanamycin. Kanamycin-resistant colonies, visible after overnight incubation at 37°C, were reisolated on kanamycin containing plates, inoculated in broth with 10 µg/ml kanamycin and transferred into mineral medium prior to amplification with chloramphenicol (5). Total DNA was isolated by treatment with proteinase K, phenol extraction and ethanol precipitation. Alternatively, the plasmid was purified from a clear lysate (19).

Hybridization experiments. DNA-DNA hybridization on filters in formamide by a macro or a microtechnique, has been described (20-21). The ^{3}HcDNA had a specific activity of 7.5 x 10^6 cpm/ µg.

For DNA-DNA hybridization in solution, sonicated plasmid or lysate DNA and ^{3}H cDNA were incubated in 0.05 - 0.1 ml 20 mM Tris-HCl (pH 7.6), 0.3 M NaCl, 1 mM EDTA for 4 hours at 65 °C, after which time aliquots were diluted into 2 ml of the buffer used for digestion with nuclease S1 (see above) together with 20 µg of denatured calf thymus DNA. The mixture was divided in two portions for the counting of the total radioactivity and the mesure of the S 1 resistance after incubation with the enzyme for 1 hour at 45°C.

Duplex formation was also determined by chromatography on hydroxylapatite as described (22). The hybridization mixture was diluted in cold 40 mM sodium phosphate buffer and absorbed at 60°C on hydroxylapatite columns (Bio gel HTP, DNA grade, batch 13579). Single strand DNA was eluted with 0.16 M phosphate and double strand DNA with 0.4 M phosphate.(> 95% of input).

RESULTS 1°/ Isolation of a bacterial clone carrying a globin DNA sequence.

Double stranded DNA was synthesized in vitro from purified 9S rabbit globin mRNA as described in the Materials and Methods section. After treatment with S1 nuclease (to remove possible single stranded tails in 5') the molecule was elongated with oligodG by terminal transferase. Purified closed circular DNA of plasmid pCR1 (derived from col E1 by the adjunction of resistance to kanamycin and containing only one EcoRI site) was treated with EcoRI. The cohesive ends were repaired with AMV reverse transcriptase before treatment with terminal transferase to elongate the molecule with oligodC "tails".

The dG-elongated globin ds-DNA and the dC-elongated plasmid DNA were annealed in molar ratio estimated to be 1:1 and mixed with $CaCl_2$ treated E. Coli cells for transformation. Kanamycin resistant transformants were then selected. The dC-elongated plasmid alone should not be able to transform since it cannot circularize. The addition of the elongated globin dsDNA should allow circularization and thus restore the transforming ability. This latter expectation is met with low efficiency since only 5 kanamycin resistant clones were observed, versus 1 in the absence of globin DNA. These five clones were analysed - as well as a dozen other clones which originated from similar experiments and which are presently being studied. In addition, it is evident from Fig. 1 that the formation of a covalent circular hybrid molecule should lead to the reconstruction of two EcoRI sites located on both sides of the globin DNA sequence.

Cultures of these bacterial clones were grown and the amplification of the plasmid was induced by chloramphenicol. Total DNA was extracted, denatured, immobilized on nitrocellulose filters, and hybridized with ^3H globin cDNA. The results (Table II) show that one of the 5 clones was positive in this test. As a control of specificity, the filters were hybridized with ^3H cDNA made from the 14SmRNA coding for the light chain of a mouse immunoglobulin. No hybridization to this heterologous cDNA was observed (Table II). The plasmid with resulted in a positive hybridization will be referred to as pCR1-βG1 (see below).

2°) Characterization of plasmid pCR1-βG1 : a) increasing amounts of total DNA extracted from cells carrying either pCR1-βG1 or the parent plasmid pCR1 were immobilized on nitrocellulose filters and incubated with a constant amount of ^3H cDNA. The results (Table III) show no hybridization to DNA containing the pCR1 plasmid. In contrast, more than 60 % of the ^3H cDNA bound to the filters carrying pCR1-βG1 DNA. Since the radioactive probe was synthesized from 9S RNA, which is a mixture of the RNAs coding for the α and β chains of globin, this result suggests extensive hybridization to the sequences for one of the two chains.

TABLE I. Transformation of E.coli cells by ds globin DNA/pCR1 DNA hybrids

DNA	Input	number of kanamycin-resistant colonies
pCR1 native	0.01 µg	1600
pCR1 cleaved with ECoRI	0.2 µg	0
pCR1-dC	0.2 µg	1
pCR1-dC/ds globin DNA	0.2 µg	5

Transformation was performed as described in Materials and Methods. For each DNA sample, 0.8 ml of $CaCl_2$-treated cells was spread over 4 kanamycin-plates.

TABLE II. Identification of a clone carrying globin DNA by filter hybridization.

source of DNA	cDNA hybridized (cpm)			
	Experiment 1		Experiment 2	
	9S (4hrs)	9S (18hrs)	9S (4hrs)	14S (4hrs)
None	7	11	18	19
pCR1	6	10	18	22
clone # 1	9	8	16	20
2	6	7	22	15
3	480	3000	485	28
4	7	10	20	22
5	6	6	18	18

Total DNA was extracted from 10 ml of chloramphenicol treated cells, denatured and immobilized onto 25 mm nitrocellulose filters out of which microfilters (diameter 5 mm) were punched . Microfilters corresponding to the various isolates were numbered and incubated together, for the times indicated, in 0.5 ml of 50 % formamide, 2xSSc at 37°C with 30 000 cpm of ^3H globin cDNA or 20 000 cpm of 14 S cDNA. The backgrounds of the counters were about 5 cpm in experiment 1 and 18 cpm in experiment 2.

b) For additional assays, the pCR1-βG1 plasmid was purified. Aliquots were sonicated, hybridized in liquid with ^3H cDNA and the products of the reaction were analysed by chromatography on hydroxylapatite. Extensive hybridization to the ^3H cDNA is, again, observed (Table IV) which indicates that the globin sequence is indeed carried by the plasmid.

c) It can be predicted that, enventhough the globin ds DNA was made from a mixture of α and β chain mRNA templates, only one of the two sequences has been incorporated into the plasmid. Purified 9S RNA was subjected to electrophoresis through acrylamide gels in the presence of 98 % formamide (Fig.2),

TABLE III. Hybridization of ^{3}H globin cDNA to increasing amounts of DNA on filters.

Amount of total DNA on filters (micrograms)	cpm hybridized to the total DNA extracted from	
	C600 (pCR1)	C600 (pCR1- βG1)
0	10	10
0.01	9	338
0.025	8	396
0.05	7	435
0.1	8	445
0.2	10	396

DNA-DNA hybridization was carried out on individual microfilters, loaded with the amounts of DNA indicated, for 18 hours at 37°C in 4 µl of 50 % formamide, 2xSSC under paraffin oil (21). There was 700 cpm of ^{3}H globin cDNA per microfilter.

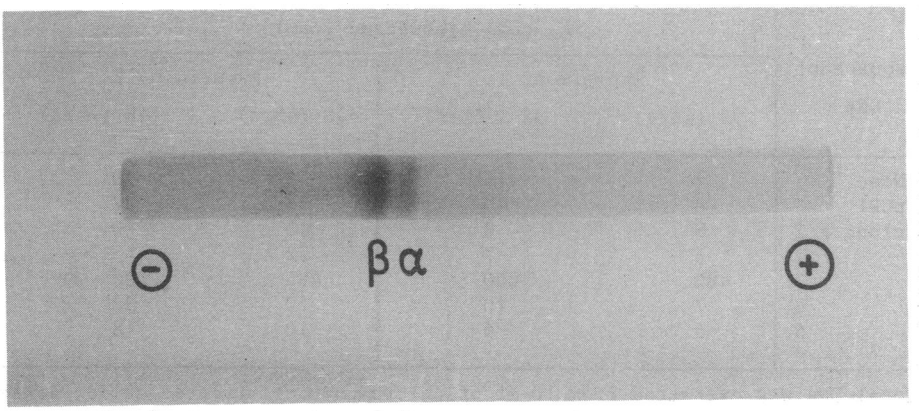

Figure 2. Acrylamide gel electrophoresis of rabbit 9 S globin mRNA (8 µg) in 98 % formamide. Staining was with Pyronin G (Serva). The slower and faster migrating RNA bands have been identified as β and α globin chain mRNA respectively (9).

and the separated α and β chain mRNAs were recovered. They served as templates for the synthesis of α and β specific ^{3}H cDNAs, which were used to analyse pCR1-βG1 further.Hybridization was carried out in liquid, and the products of the reaction analysed by chromatography on hydroxylapatite. The results show that pCR1-βG1 carries a sequence specific of the β chain of rabbit globin mRNA (Table IV). The melting temperature of the hybrids formed between β globin ^{3}H cDNA and purified pCR1-βG1 DNA was measured (Fig.3). The observed T_m is high (86°) and compares favourably with

TABLE IV. Analysis of hybrids by chromatography on hydroxylapatite (HAP) and by digestion with nuclease S 1.

	Unlabelled DNA (or RNA)		mRNA template for ^{3}H-cDNA globin 9S	α chain	β chain	14 S
Part I HAP	pCR1	0.16M	10131	5450	3860	3998
		0.4 M	600(5.6%)	849(13.9%)	611(13.6%)	197(4.7%)
	pCR1-βG1	0.16M	5086	6183	2952	5820
		0.4 M	3196(38.5%)	1723(21.8%)	5319(64.3%)	1108(15.9%)
Part II Nuclease S1	none	- S1	--	--	879	729
		+ S1	--	--	27(3%)	24(3.2%)
	pCR1	- S1	2355	--	1740	1701
		+ S1	84(3.5%)	--	94(5.4%)	41(2.4%)
	pCR1-βG1	- S1	2448	2346	2334	1561
		+ S1	217(8.8%)	174(7.4%)	471(20.1%)	56(3.5%)
	9 S mRNA	- S1	1680	--	--	--
		+ S1	1330(79%)	--	--	--
	14S mRNA	- S1	--	--	--	1719
		+ S1	--	--	--	896(52%)

Hybridisations (4 hours , 65°C) and assays for hybrid formation were as described in Materials and Methods. In part I, the results are expressed as cpm in the single-strand (0.16M PO_4) and duplex (0.4 M PO_4) fractions and as % of the ^{3}H-cDNA found as duplex. In part II the cpm in controls and in S1 nuclease-treated samples, are presented, with the % of ^{3}H-cDNA resistant to S1.

reported data on the melting of globin cDNA hybridized to homologous cellular DNA (22).

(d) Hybrids formed between pCR1-βG1 and ^{3}H cDNA were also analysed by digestion with nuclease S1. The results of this experiment and the appropriate controls are shown in Table IV. It can be seen that the % of cDNA

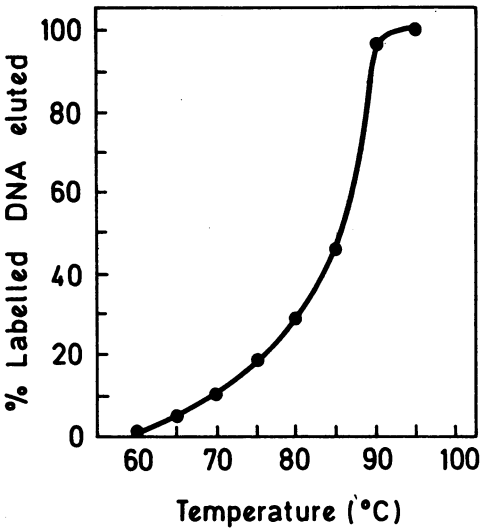

Figure 3. Thermal denaturation of globin β chain ^{3}H-cDNA/plasmid duplexes. After hybridisation, the sample was loaded onto a hydroxylapatite column in 0.16 M PO_4 buffer. After elution of single stranded DNA, the temperature was raised and elution continued with 0.16 M PO_4 buffer. Radioactivity in TCA-precipitable material was measured in each fraction and expressed as per cent of the total radioactivity eluted from 65° to 95° (=65% of total ^{3}H-cDNA applied to the column).

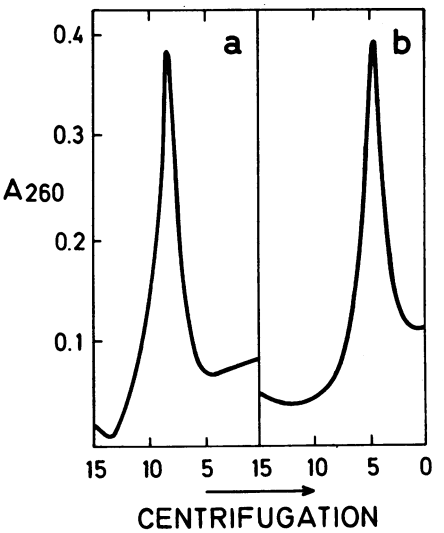

Figure 4. Cleavage of close circular pCR1-βG1 plasmid DNA by EcoR1. (a). 10 µg of purified pCR1-βG1 plasmid DNA was incubated for 1 hour at 37° (90 mM Tris HCl, pH 7.6, 10 mM $MgSo_4$) in the presence of EcoR1, made 10 mM EDTA and centrifuged in a 5-20% sucrose gradient (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1 M NaCl) at 20° and at 45 000 rpm for 130 min (SW 50.1 Spinco rotor) (b). Control undigested sample.

found resistant to S1 digestion corresponds to about 1/3 of the radioactivity measured in duplex form with hydroxylapatite. Since the size of the ^{3}H cDNA used corresponds to about 300 to 400 nucleotides (17), it can be estimated that the length of the integrated sequence of globin DNA might be about 100 to 150 nucleotides.

e) Does pCR1-βG1 carry EcoRI sensitive sites ? Purified plasmid DNA was analysed by centrifugation in neutral sucrose gradients with or without prior exposure to the EcoRI nuclease. As shown in Fig.4, treatment with the nuclease causes complete conversion of the rapidly sedimenting closed circular form to the more slowly sedimenting linear form. From this, it can be concluded that pCR1-βG1 carries at least one site sensitive to EcoRI endonuclease.

DISCUSSION

In this report, we describe the integration of a specific mammalian DNA sequence into a bacterial plasmid. Double stranded DNA was synthesized *in vitro* from purified rabbit globin 9S RNA. The preparation of this globin ds-DNA is described in details elsewhere (17), but it should be mentionned that several steps could not be entirely controlled. The ds-DNA molecules were shorter, on the average, thant the cDNA used as template. This might reflect either uncomplete synthesis of the second DNA strand or endonucleolytic activity in some of the enzymes used (such as the commercial "Klenow" fraction of *E.coli* DNA polymerase or nuclease S1). A second uncontroled step was the elongation of the globin ds-DNA with terminal transferase. Eventhough the ds-DNA had been treated with S1 nuclease to provide the exposed 3'OH groups necessary for terminal addition, there was no evidence that the latter occured in a symetrical fashion on all molecules.

The strategy for integration was such that the dC-elongated kanamycin resistant plasmid could not form circles unless paired with the dG-elongated globin ds-DNA. Upon transformation of *E.coli* to kanamycin resistance, this should confer a positive selection in favour of those plasmids in which foreign DNA was inserted. In this experiment, however, the positive transformation events were rare, perhaps because the globin ds-DNA had not been elongated symetrically. We obtained five clones, out of which only one was found to be positive with respect to hybridization with globin ^{3}H cDNA. These clones, as well as other globin-positive clones obtained in other similar experiments are currently under study.

The positive clone was shown to harbour a plasmid (named pCR1-βG1) with the following properties (a).Excess plasmid DNA, in liquid

or on filter, hybridizes extensively with ^3H globin cDNA. (b). Little hybridization is obtained with ^3H cDNA made from globin α chain mRNA, while extensive hybridization is obtained with ^3H cDNA made from β chain mRNA, and the Tm of the hybrids formed is high. (c). The DNA of the plasmid can protect about one third of the hybridized ^3H cDNA probe from digestion by nuclease S1. (d). The plasmid DNA is susceptible to the EcoRI nuclease.

The specificity of the hybridization reaction was verified in a number of ways. The reaction was carried out under several conditions (in liquid or on filters) and different assays were used. DNA from the parent plasmid pCR1 does not hybridize to ^3H globin cDNA. pCR1-βG1 DNA shows little or no hybridization with ^3H cDNA made from another eukaryotic mRNA (the 14S RNA of a mouse immunoglobulin light chain) or from globin α chain RNA. In contrast it hybridizes to cDNA made from globin β chain mRNA. The Tm of the hybrids formed (86° in 0.16 M phosphate buffer) is high, showing good matching of the duplex.

The homogeneity of the 9S RNA used as a template for the synthesis of the globin ds-DNA or of the ^3H cDNA probe has been estimated by means of RNA-cDNA hybridization kinetics, and found to be higher than 80 % (17). Since over 60 % of total or β globin cDNA could be hybridized to pCR1-βG1, it is unlikely that the sequence incorporated into the plasmid represents the DNA transcript of a contaminant RNA species.

From these observations, we conclude that pCRI-βG1 carries a DNA sequence specific of the β chain of rabbit globin.

The length of this sequence can only be estimated indirectly at this stage. The sequence is longer than that required to ensure a Tm of 86°C in 0.16 M phosphate buffer, as monitored by hydroxylapatite chromatography. The ^3H globin cDNA used as a probe has an average size of about 300 to 400 nucleotides. Comparison of hybridization assays with nuclease S1 and hydroxylapatite suggest that pCR1-βG1 carries about one third of the sequence of the probe, or 100 to 140 nucleotides. This figure could be underestimated : the portion of the globin sequence corresponding to the 5' region of the mRNA is not detected in this assay, since the ^3H cDNA probe represents less than 2/3 of the length of the 9S mRNA sequence. If the inserted globin ds-DNA has resulted from the replication of longer unlabelled cDNA molecules, part of the sequence carried by the plasmid could remain undetected.

The strategy of integration was devised in such a way as to reconstruct EcoRI sites on both sides of the integrated sequence (Fig.1). Our preliminary data indicate that pCR1-βG1 carries at least one EcoRI site (Fig.4). Work is in progress to determine whether this site lies within

the integrated sequence, or whether EcoRI sites have been reconstructed.

The principle of the method described here is general and could be applied to the integration, into plasmid or phage DNA, of other eukaryotic DNA sequences synthesized in vitro from viral or cellular RNAs. An interesting aspect of the procedure is that, starting from a non homogeneous preparation of RNA, one can achieve the purification of a given sequence upon integration and cloning. This is illustrated here by the cloning of a β globin sequence from a mixture of α and β sequences.

An important and obvious limitation of the method which we describe is that only a portion of a given gene, rather than the entire gene and the neighbouring regions, is inserted. However, the fact that milligram amounts of the plasmid carrying a rabbit globin gene sequence can be easily obtained, makes available large amounts of a specific DNA probe. This will increase the sensitivity and feasability of hybridization experiments to detect trace amounts of complementary RNA or DNA sequences, and should be of importance, for instance, in studies of erythroid differenciation or in the analysis of gene expression in native and reconstituted chromatin. Also, it should now be possible to purify large amounts of α and β globin mRNA by preparative hybridization to DNA of the new plasmid. Finally, and, perhaps, most important, the use of the recombinant plasmid might make it possible to purify, by hybridization, the two complementary strands of the rabbit DNA fragments produced by restriction endonucleases and containing globin genes. After reassociation, the specific fragments could be inserted into plasmid or phage DNA for the analysis of the entire gene. It is hoped that the method presented in this paper will help in the analysis of other eukaryotic genes.

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