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Mapping of RNA by a modification of the Berk-Sharp procedure: the 5' termini of 15 S β-globin mRNA precursor and mature 10 S β-globin mRNA have identical map coordinates

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ABSTRACT

We have used a modification of the Berk-Sharp technique to determine that the 5' termini of the mouse 15 S β-globin precursor and the mature mRNA have identical map coordinates. The modification involves the use of 5' (or 3') terminally labeled probes; it allows the detection of the precursor in the presence of excess mature mRNA.

INTRODUCTION

A 15 S β-globin-specific RNA has been identified as a precursor of mature 10 S β-globin mRNA in dimethylsulfoxide-induced erythroleukemic mouse cells (1,2), spleen cells of anemic mice (3), immature red blood cells of the duck (4,5) and mouse fetal liver (6-8). It has been proposed that an even longer RNA species precedes the 15 S β-globin precursor (2,9). However, this suggestion has yet to be confirmed.

Tilghman et al. (10,11) and Jeffreys and Flavell (12) have shown that the coding sequences of the β-globin genes, both in mouse and in rabbit, are subdivided into three blocks by a smaller and a larger non-coding region (intron), located at the positions corresponding to amino acids 30/31 and 104/105, respectively (13,14). The 15 S β-globin precursor of the mouse contains transcripts of the introns as shown by electron microscopy, or digestion of hybrids between 15 S RNA and β-globin cDNA hybrids followed by analysis of the products (11,15,16). Restriction mapping of a cloned partial DNA copy of 15 S RNA (17) has shown that the positions and sizes of the introns coincide with those

described in the mouse chromosomal β-globin major DNA fragment, βG-1, cloned by Tilghman et al. (10).

The 15 S precursor has been described as having a length of about 1500 nucleotides by Curtis et al. (18); however, Ross and Knecht (7) report a length of around 1860 nucleotides. Since the distance between the sites coding for the first and the last nucleotide of the β-globin major mRNA is about 1400 nucleotides (13), the question arises as to whether the 15 S precursor comprises additional 5' or 3' terminal sequences not found in the mature β-globin mRNA.

In this paper we use a modification of the Berk-Sharp procedure (19) to show that the 5' termini of 15 S precursor and mature β-globin mRNA of the mouse map at the same position on β-globin chromosomal DNA. A preliminary account of this work has been given (20).

MATERIALS AND METHODS

Cloned mouse globin DNA.

The cloned EcoRI fragment of mouse β-globin major chromosomal DNA MβG-2 (isolated by Tilghman et al., (10)) was transposed from λ into the EcoRI site of plasmid pBR322 (14). This recombinant DNA (Z-pBR322/MchrβG-36) was propagated in E.coli X1776, and purified by a combination of the methods of Guerry et al. (31) and Currier and Nester (32).

Preparation of 5'-^{32}P-labeled probes.

50 µg of Z-pBR322/MchrβG-36 were digested with BamHI (New England Biolabs, Beverly, Mass.) and EcoRI (a gift from W. Boll) and extracted with phenol. To prepare labeled tracer for the further steps, one µg of this digested DNA was treated for 30 min at 37°C with 0.003 units of calf intestine alkaline phosphatase (Boehringer, Mannheim) in 15 µl of 10 mM Tris-HCl (pH 9.5), 1 mM spermidine, 0.1 mM EDTA. The alkaline phosphatase was inactivated by heating for 60 min at 65°C. The dephosphorylated DNA was incubated with 75 units of polynucleotide kinase (P-L Biochemical Inc.) for 20 min at 37°C in 50 mM Tris-HCl (pH 9.5), 10 mM $MgCl_2$,

5 mM dithiothreitol, 1 mM spermidine, 0.1 mM EDTA, 0.5 μM γ-^{32}P-ATP (NEN, 2000 Ci/mmole). The reaction was stopped by adding ammonium acetate to a concentration of 0.2 M. The DNA was recovered by chromatography on a 8 x 0.5 cm column containing 1.6 ml Sephadex G-100 layered on 0.1 ml Chelex-100, in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA (TNE) buffer. About 150'000 dpm of this labeled tracer DNA were added to the unlabeled, restricted DNA, and the mixture was subjected to electrophoresis on a 0.7% agarose gel containing 89 mM Tris-borate (pH 8.35), 1.8 mM EDTA (TBE buffer). Electrophoresis was at about 4 mA/cm^2 for 14 h. The gel was autoradiographed and the film used as a template to cut out the 1800 bp Bam-Eco fragment. The gel slice was minced and the DNA eluted by rocking with gel elution buffer (0.15 M NaCl, 50 mM Tris-HCl (pH 8), 5 mM EDTA). The DNA was purified by adsorption to DEAE cellulose (Cellex D, Bio-Rad, 0.05 ml bed volume/μg DNA) in gel elution buffer, followed by batch elution with 50 mM Tris (pH 8), 2 mM EDTA, 1.5 M NaCl, then ethanol precipitated and taken up in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA.

The 1800 bp Bam-Eco fragment was either directly dephosphorylated and rephosphorylated using γ-^{32}P-ATP (1 μM, specific activity 2000 Ci/mmol), or first digested with either BspI (generously provided by Dr. A. Kiss) or AluI (New England Biolabs), and then 5'-terminally labeled as above. The 5'-labeled BspI and AluI digests were fractionated by electrophoresis through a 5% polyacrylamide gel in 36 mM Tris-phosphate buffer (pH 7.8) (33). The 340 bp AluI fragment and the 210 bp BspI fragment were cut out, eluted, purified and ethanol precipitated as above.

The minus strand of the ^{32}P-labeled 340 bp AluI fragment was purified as follows: 1.9 μg mouse β-globin mRNA (a gift from P. Curtis, repurified by chromatography on oligo(dT) cellulose as described below) were hybridized to approximately 100 ng of heat-denatured 340 bp ^{32}P-labeled AluI fragment in 80% formamide, 0.2 M NaCl, 20 mM PIPES (pH 6.4), 0.5 mM EDTA, for 4 h at 57°C. The hybridization mixture was diluted ten-fold with loading buffer (100 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10 mM EDTA, 0.1% sodium

dodecylsulfate, 0.02% polyvinyl sulfate) and passed through an oligo(dT) cellulose (Sigma) column (50 µl bed volume). The column was washed with 10 volumes of loading buffer, by which time no more radioactive DNA was eluted. The hybrid was then eluted with 10 mM Tris-HCl (pH 7.5), 0.1% sodium dodecylsulfate. The mRNA was hydrolyzed in 0.5 M NaOH for 2 h at 23°C. After neutralization with glacial acetic acid, the solution was diluted tenfold with 0.3 M sodium acetate buffer (pH 7.5), 10 µg of yeast carrier RNA were added, and the nucleic acid was precipitated with ethanol. The sample was dissolved in 20 µl of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA.

Hybridization assay.

The 5'-labeled probe DNA (approximately 5 ng, 1500-5000 dpm) and 10 µg of yeast carrier RNA were placed in a 1.5 ml Eppendorf centrifuge tube and dried in a stream of nitrogen or under low vacuum over NaOH pellets. The residue was taken up in 10 µl of 80% (w/v) formamide (Fluka, puriss, p.a., > 9% pure) and denatured in a tightly closed tube in a boiling water bath for 15 min. (We have recently noted (H. Weber, personal communication) that under these conditions long DNA is degraded; it may thus be preferable to shorten the period of heating). The RNA sample along with 2 µl of 5-fold concentrated hybridization buffer (2 M NaCl, 0.2 M PIPES (pH 6.4), 0.005 M EDTA) was dried down as above. The solution of denatured DNA (10 µl) was then added to the dried RNA and incubated at 57°C for 14-18 h under a drop of paraffin oil. The mixture was transferred into 100 µl of chilled S_1 buffer (0.25 M NaCl, 0.03 M sodium acetate buffer (pH 4.6), 0.001 M $ZnSO_4$, 20 µg/ml denatured salmon sperm DNA (Calbiochem AG, Lucerne)). Next, 55 units of nuclease S_1 (11'000 units/ml; prepared by A. Schamböck by the method of Wiegand et al. (34)) were added and digestion was carried out for 40 min at 30°C. Finally, 10 µg of yeast carrier RNA and 2 vol of ethanol were added. The precipitated nucleic acid was taken up in 5 µl of loading solution (0.05% bromphenol blue, 0.05% xylene cyanol, 1 mM EDTA, 90% (v/v) formamide), heated for 2 min in boiling water and electrophore-

sed on a 5% polyacrylamide gel for 3.5 h at 17 mA/cm^2, in TBE buffer containing 7 M urea. The gel was autoradiographed with Fuji RX (Safety) X-ray film and an intensifying screen at -70°C for 7-28 days.

RESULTS

(1) Mapping of RNA by a modification of the Berk-Sharp procedure.

In the original Berk-Sharp procedure (19), RNA is hybridized to a denatured, ^{32}P-labeled DNA restriction fragment under conditions which disfavor DNA-DNA renaturation (21). The mixture is then treated with S_1 nuclease and the length of the protected fragment is determined by alkaline agarose gel electrophoresis (22). This procedure allows the detection of an RNA complementary to part or all of the labeled probe but does not distinguish whether a plus or a minus strand RNA was present. If only part of the probe is protected, further analyses are required to establish which is the protected part.

In a modification of the Berk-Sharp procedure, we utilize a DNA restriction fragment ^{32}P-labeled at either the 5' or 3' terminus of one strand. The unlabeled RNA is hybridized with an excess of the labeled DNA, the concentration of which determines the reaction rate. The reaction is carried out in 80% formamide, 0.4 M NaCl, which, at the appropriate temperature, largely prevents DNA-DNA hybridization (21). If the 5' terminus is overhanging, as in the case of EcoRI or BamHI fragments, no background is found even if DNA-DNA self-annealing occurs, because of the susceptibility to nuclease S_1 of the labeled end. In the case of flush ends or of 3' overhanging ends self-annealing, when it occurs, leads to a labeled band corresponding to the double-stranded probe. We have, on occasion, separated the strands of the labeled DNA fragment (either on strand separation gels or, when possible, by hybridizing the DNA to mRNA and isolating the hybrid on oligo(dT) cellulose) and used the appropriate radioactive single-strand as a probe. When this is done, hy-

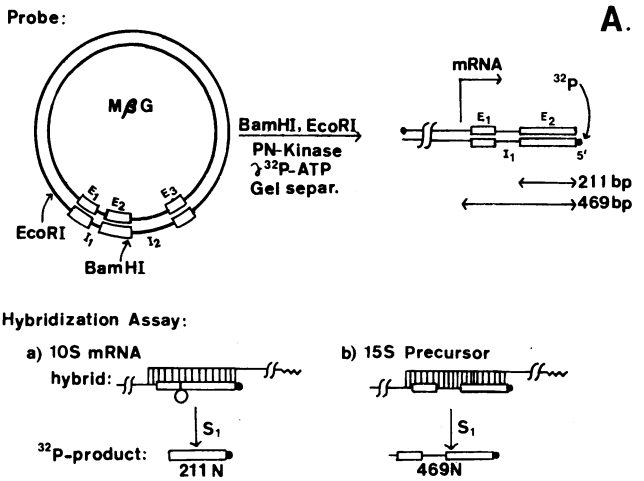


Figure 1. Effect of S_1 digestion conditions on the length of the protected probe in the modified Berk-Sharp assay. A. Diagram of experimental strategy. Z-pBR322/MchrβG-36 DNA (MβG) was digested with BamHI and EcoRI, and 5'-terminally labeled with ^{32}P. The 1800 bp fragment, the Bam end of which is located in exon 2 of the β-globin gene, was isolated by agarose gel electrophoresis. 10 S globin mRNA will hybridize to this probe to protect a labeled fragment 211 nucleotides long; 15 S precursor RNA will protect a fragment 469 nucleotides long. E, exon; I, intron. B. Autoradiogram of the electropherogram of the protected fragments. About 100 ng of Bam-Eco probe (1×10^5 dpm/μg DNA) were hybridized for 14 h to 10 ng of 10 S β-globin mRNA in 100 μl. Aliquots (10 μl) were diluted with 100 μl of S_1 buffer and treated at the temperatures and with the amounts of S_1 indicated below. 200 dpm of 5' end-labeled 104-bp BspI fragment of pBR322 DNA were then added to each sample to monitor recoveries. Each sample was precipitated with ethanol, washed with 80% ethanol, the precipitate was dissolved in 5 μl of dye mix and analyzed by polyacrylamide gel electrophoresis. Experimental details are described in Materials and Methods. Lanes a, g and m, pBR322 DNA cleaved with BspI and 5' terminally labeled (marker); lanes b-f, digestion with 500 units/ml of S_1 at 16°C, 23°C, 30°C, 37°C and 44°C, respectively; lanes h-l, digestion at 30°C, with 125, 250, 500, 1000 and 2000 units/ml of S_1, respectively.

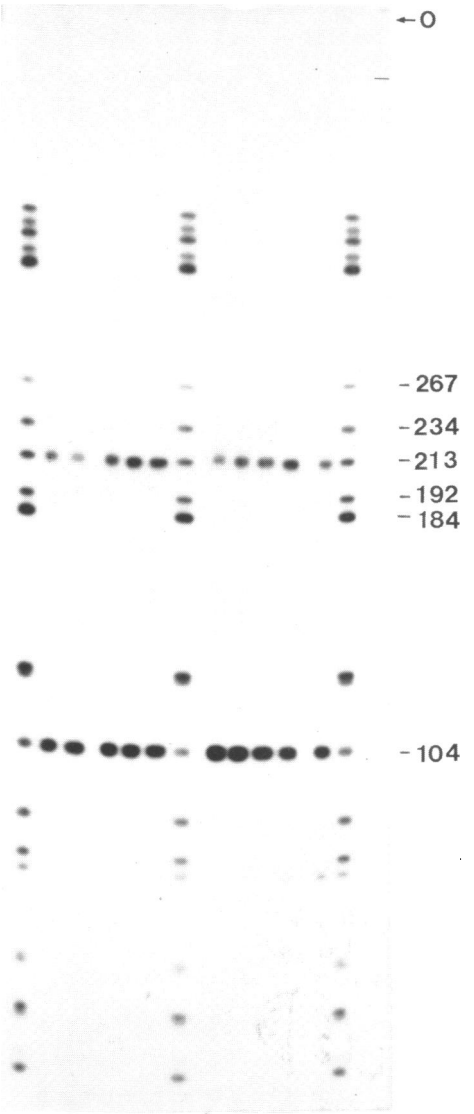


Fig. 1B

bridization can be carried out in the absence of formamide, and the narrow temperature window which permits DNA-RNA but not DNA-DNA hybridization need no longer be sought.

The unhybridized DNA is digested with nuclease S_1. Since it was thought that some trimming of the ends might occur, the effect of a wide range of temperatures and enzyme concentrations was tested, using mouse globin mRNA hybridized to the DNA probe shown in Fig. 1. Under the standard reaction conditions (500 units S_1/ml, 40 min, 30°C) a single major band corresponding to 213 nucleotides was observed. This corresponds, within two nucleotides, to the distance between the ^{32}P-labeled BamHI-generated terminus on the minus strand to the end of the region protected by the mRNA. Digestion at higher temperature led to a slight increase in mobility of the probe, corresponding to a shortening by 1-2 nucleotides while after digestion at lower temperatures the major band moved slightly more slowly, indicating the presence of 1-2 additional nucleotides; in addition, several fainter bands of lower mobility appeared, due to incomplete digestion of the hybridized probe. Similar effects were observed on varying the enzyme concentration. It would seem from these experiments that while underdigestion can give rise to spurious bands, overdigestion does not cause the length of the protected probe to diminish by more than a few nucleotides. Most likely, overdigestion does not lead to bands of substantially increased mobility because as trimming of the ends of the hybrid occurs, the label is lost. In fact, after digestion at 52°C no fragment could be detected at all (data not shown). Nevertheless, if the 3' terminus of the hybridized probe is in an AT-rich region while the labeled 5' end is GC-rich, then unilateral degradation could, in theory, lead to an artefactually shortened probe.

Analysis of the DNA fragment is carried out by polyacrylamide gel electrophoresis in 7 M urea, to allow the same resolution achieved in sequencing gels. In fact, accurate size calibration may be obtained by the use of probe samples degraded by the Maxam-Gilbert technique (23) (see below).

(2) The map position of the 5' end of the 15 S mouse β-globin precursor RNA.

In order to map the 5' terminus of 15 S β-globin precursor RNA on the cognate chromosomal DNA, in the possible presence of contaminating 10 S RNA, the approach outlined in Fig. 2A was taken. Cloned mouse β-globin chromosomal DNA Z-pBR322/MchrβG-36 has an AluI site in the small intron. A fragment extending from this AluI site to the AluI site approximately 340 bp upstream was ^{32}P-labeled at the 5' termini and the minus strand was isolated by hybridization to β-globin mRNA followed by oligo(dT) cellulose chromatography. The 5'-^{32}P-labeled terminus of this probe can only be protected by 15 S precursor RNA, since the 10 S mRNA has no sequences complementary to the introns. The ^{32}P-labeled, single-stranded DNA was hybridized to a preparation of 15 S β-globin precursor RNA, treated with endonuclease S_1, and denatured. A high resolution size marker was obtained by subjecting a sample of the single-stranded probe to the Maxam-Gilbert degradation procedure for A and G cleavage. Fig. 2B shows an autoradiogram of the protected probe, the size marker and a mixture of the two analyzed by polyacrylamide gel electrophoresis in 7 M urea.

Experiments by Pugatsch and Weber (personal communication) have shown that the mobility of a fragment generated by Maxam-Gilbert cleavage does not differ from that of a fragment of equal length generated by S_1 cleavage, although the former has a 3' terminal phosphate, while the latter does not. The protected probe fragments comigrate with two marker fragments extending to 4 and 5 nucleotides, respectively, before the "cap nucleotide" (cf. Fig. 2C). Since it is known that the 5' terminal cap of the 15 S RNA precursor has the probable sequence G^{7m}pppApC (18), and there is no other A-C sequence in the vicinity, we suspected that the probe fragment was protected against S_1 cleavage for a few nucleotides beyond the 5' terminus of the RNA, perhaps because of steric hindrance by the cap group.

To test this conjecture, a new probe was prepared that allow-

ed a direct comparison between the 15 S precursor and the mature 10 S mRNA, both of which are capped (18). This was a 210 bp BspI fragment extending from 76 bp before to 133 bp beyond the 5' terminus of the 10 S mRNA (Fig. 3A). Since the 5' end of the minus strand of this fragment lies upstream from the small intron, it will be protected by both the 15 S and 10 S RNAs. Fig. 3B shows that both RNAs protect a labeled 138-nucleotide fragment of the probe. Thus, the 5' termini of the 15 S precursor and the mature 10 S mRNA map at the same location. Furthermore, the size of the protected fragment is, in both cases, five nucleotides longer than expected. This is the same effect observed in the experiment of Fig. 2 and is consistent with incomplete digestion of the 3' end of the hybridized probe.

A possible objection to the interpretation of the last experiment arises if the 15 S RNA preparation contained a high proportion of 10 S RNA, because the band in the 15 S lane could then be attributed to protection by the contaminating 10 S RNA. Therefore, the relative content of 10 S and 15 S RNA was determined by a probe allowing discrimination between these two species. This is an 1800 bp BamHI EcoRI fragment of chromosomal mouse β-globin DNA mapping as shown in Fig. 4. The labeled 5' end of the minus strand lies 208 bp downstream from the 3' bor-

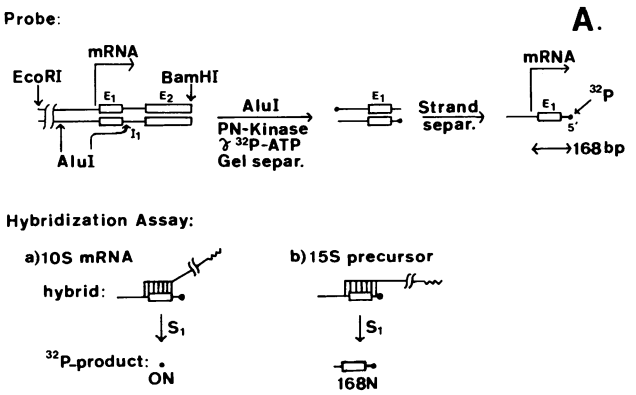
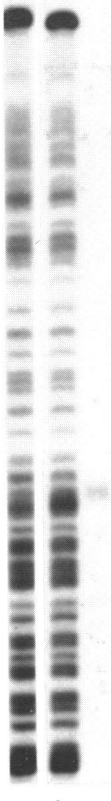


Fig. 2A



B

C

mRNA

PLUS T T G C T C C T C A C A T T T

MINUS A A C G A G G A G T G T A A A

Pu Pu Py Pu Pu Pu Pu Pu Pu Py Pu Py Pu Pu Pu

* *

Figure 2. Mapping the 5' terminus of 15 S β-globin precursor RNA by hybridization to a precursor-specific probe. A. Diagram of experimental strategy. The 1800 bp Bam-Eco fragment of Z-pBR322/MchrβG-36 DNA (cf. Fig. 1) was cleaved with AluI, then 5' terminally labeled and the products separated by polyacrylamide gel electrophoresis. The 340 bp fragment, one end of which is located in the small intron of the β-globin gene, was isolated. To separate the strands, the fragment was denatured and hybridized to mouse globin mRNA followed by oligo-(dT) cellulose chromatography. The 15 S precursor RNA will hybridize with this probe to yield a labeled, protected DNA fragment 168 nucleotides long, but mRNA will not protect the labeled terminus, and will not give rise to a signal. B. Autoradiogram of the electropherogram of protected fragments. Approximately 5 ng of 240 bp AluI probe (4 x 10^5 dpm/μg) were hybridized for 14 h at 57°C to 0.5 ng 15 S β-globin precursor RNA (fractions 13 and 14 of the preparation described in ref. 11) in 10 μl. Hybrids were trimmed with S_1 and the probe analyzed as described in Materials and Methods. Lane a, fragments resulting from A+G cleavage, according to Maxam and Gilbert (23), of the 340 bp AluI DNA probe; lane b, fragments as in lane a plus probe protected by 15 S precursor RNA; lane c, probe protected by 15 S precursor RNA. C. Sequence of DNA region coding for the 5'-end of mouse β-globin mRNA, compared to sequence deduced from A+G fragments in lane a. Asterisks designate the nucleotides of the probe (G and A bands on the ladder) which are eliminated to give rise to the two fragments with lengths equal to those of the protected probe. The arrows indicate the two 3' terminal nucleotides of the marker fragments which have the same mobility as the protected probe fragments.

der of the small intron of the β-globin gene. Because the three nucleotides preceding the small intron are repeated at its end, the 10 S mRNA could protect 211 nucleotides of the probe while 15 S RNA, which contains the entire intron sequences, will protect a fragment of about 470 nucleotides. Fig. 4 shows that the 15 S RNA preparation used in these experiments protects two fragments of about 465 and 213 nucleotides. The ratio of radioactivity in the upper to that in the lower band is about 1:2. This result shows that although the 15 S RNA preparation is not pure, it contains enough 15 S RNA relative to 10 S RNA that two bands should be detected in Fig. 3 if 10 S and 15 S RNA started at different points. The fact that only one band appears shows that the 10 S and 15 S RNAs protect the same size fragment.

DISCUSSION

(a) Application of the modified Berk-Sharp assay.

The use of a DNA probe labeled at one terminus (5' or 3' (24)) in the Berk-Sharp assay allows the determination of the length of an RNA from the site corresponding to the labeled ter-

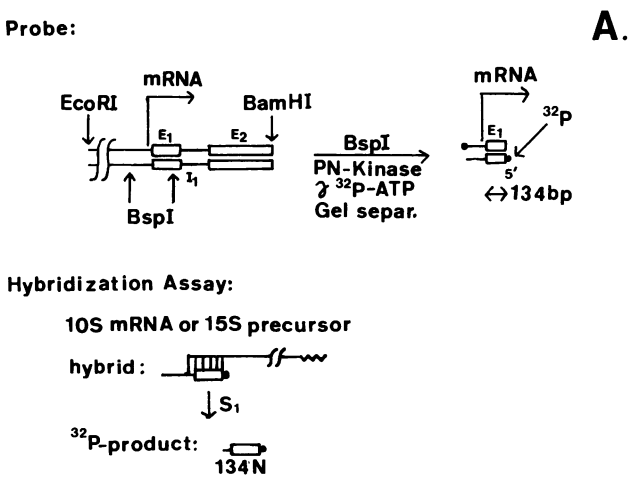


Fig. 3A

Figure 3. Mapping the 5' ends of 10 S β-globin mRNA and 15 S precursor RNA with a probe responding to both species. A. Diagram of experimental strategy. The 1800 bp Bam-Eco fragment of Z-pBR322/MchrβG-36 DNA (cf. Fig. 1) was cleaved with BspI and MboII. The fragments were 5'-terminally labeled and the 210 bp fragment shown in the figure purified by polyacrylamide gel electrophoresis. (MboII served to cleave a second BspI fragment of almost equal length to the one sought). Both 15 S precursor RNA and mature mRNA will protect the 5' end of the probe. B. Autoradiogram of the electropherogram of protected probes. Approximately 5 ng of 210 bp BspI probe ($\sim 4 \times 10^5$ dpm/μg) were hybridized for 17 h at 57°C to either 2 ng 10 S β-globin mRNA or 0.5 ng 15 S β-globin precursor RNA in 10 μl reaction volume. Hybrids were trimmed and the probe analyzed as described in Materials and Methods. Lanes a and e, pBR322 DNA cleaved with BspI and 5' terminally labeled (marker); lane b, fragment protected by 10 S mRNA; lane c, fragment protected by fractions 11 and 12 of the 15 S RNA preparation described in ref. 11; lane d, probe cleaved with HindII, as additional marker.

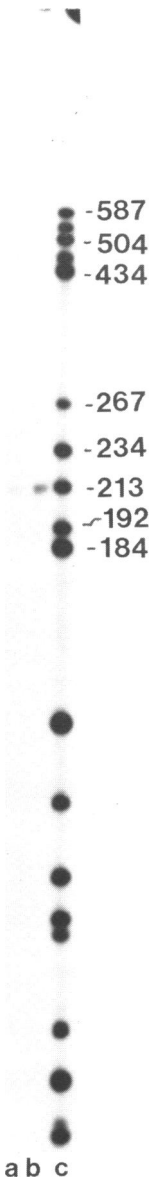


Figure 4. Determination of the 10 S β-globin mRNA contamination in the preparation of 15 S precursor RNA. Experimental strategy was as outlined in Fig. 1A. Approximately 10 ng of Bam-Eco probe (~2 x 10^5 dpm/μg DNA) were hybridized for 18 h at 57°C to either 1 ng 10 S β-globin mRNA or 0.5 ng of 15 S β-globin precursor RNA preparation used in the experiment of Fig. 3 in 10 μl reaction volume. Hybrids were trimmed and the probes analyzed as described in Materials and Methods. Lane a, fragments protected by the 15 S preparation; lane b, fragment protected by 10 S β-globin mRNA; lane c, pBR322 DNA cleaved with BspI and 5' terminally labeled (marker).

minus of the probe to the end of the RNA, or to the point where the sequences of the RNA and the probe become non-complementary. When chromosomal DNA of a split gene is hybridized to mature mRNA the intronic DNA sequences pertaining to the intron are

looped out and removed by nuclease S_1 digestion (19), allowing the determination of the distance from the 5' end of the probe to the edge of the intron. In the converse experiment, when an intron-containing mRNA precursor is hybridized to the cognate cDNA, the looped-out intronic RNA sequences are excised and, at least in the case of mouse β-globin precursor, most if not all of the DNA molecules are cleaved at the junction point between the two exons under the standard conditions of S_1 nuclease digestion (data not shown). Results reported by Kamen suggest that at least in some cases cleavage of the DNA does not occur (25).

The modified method, in contrast to the original one, yields map distances directly. Quite recently, the use of the modified approach to this purpose has been reported by Berget and Sharp (26) and Tsujimoto and Suzuki (27). However, the use of probes ^{32}P-labeled at one terminus has several additional advantages. (1) It is possible to distinguish plus from minus strand transcripts by using appropriate probes. For example, the rabbit chromosomal DNA-containing plasmid Z-pCRI/RchrβG-1 DNA was cleaved at the unique BamHI site, 5'-terminally labeled with ^{32}P, and cleaved with SalI. The resulting two fragments, one of which was labeled on the plus, the other on the minus strand, were separated by agarose gel electrophoresis. These probes were hybridized to the *in vitro* transcription product generated by E.coli RNA polymerase with the chromosomal β-globin DNA hybrid Z-pCRI/RchrβG-1 plasmid as template. Hybridization with the plus strand, but not the minus strand probe, led to a protected, labeled fragment, indicating the presence of minus, but not plus strand β-globin RNA (A. van Ooyen, unpublished results). (2) The choice of a probe with the appropriate labeled cleavage site may allow the clear distinction of homologous mRNAs from different species. For example, rabbit but not mouse β-globin mRNA has a BglII recognition site (immediately following the last exon). Thus, hybridization of rabbit, but not mouse, β-globin mRNA to a rabbit chromosomal β-globin DNA probe labeled at the BglII site protects a radioactive 134 nucleotide fragment (28). Conversely, the mouse β-globin mRNA, but not its rabbit counterpart, contains an AluI

recognition sequence at the position of amino acids 44/45, allowing its specific detection by an analogous assay (28). (3) As shown in the results section, it is possible to detect specifically a mRNA precursor or map its termini in the presence of mature RNA, by using a probe with a labeled terminus within an intron. The ratio of precursor to mature RNA can be determined by choosing a probe with a 5' labeled end located in an exon downstream from an intron or, conversely, with a 3'-labeled end upstream from an intron; the two RNA species will then protect probe fragments of different length, the amounts of which can be quantitated on one and the same gel (cf. Fig. 4). We generally assume that the shorter fragment indicates the absence of the intron (24,28); this assumption is not strictly warranted, inasmuch as absence of only part of the intron could lead to the same result.

(b) Some qualifications of the method.

In general, the map distances obtained by the modified Berk-Sharp procedure seem accurate to within a few nucleotides. As shown in the Results section, the 5' terminus of the mouse β-globin mRNA maps 138 rather than 134 nucleotides upstream from the 5'-labeled end of the probe, perhaps due to steric hindrance by the cap. In the experiment in Fig. 1, the protected fragment was within two nucleotides of the predicted length.

We have noted (Fig. 1) that underdigestion of the hybrid may yield discrete, overlong DNA fragments. The fact that such fragments do not appear in controls without added mRNA, i.e. under conditions where the 5' proximal part of the probe is not protected, may lead to the erroneous conclusion that longer mRNA molecules exist. In case of doubt it is therefore advisable to carry out digestions under conditions of increasing stringency.

We observed a further artefact when using a DNA probe (PβG) in which the coding sequence was preceded by an interrupted stretch of dT residues (29). In this case, hybridization of rabbit β-globin mRNA with the probe, which was 5'-terminally labeled at the BamHI site, yielded three labeled fragments, the ex-

pected one of 335 nucleotides, and two more, of lower intensity and longer by about 30 and 60 nucleotides, respectively. These longer fragments most likely arose by protection of the interrupted oligo(dT) sequence of the probe by the poly(A) of mRNA, since they could be eliminated by adding an excess of poly(U) to the hybridization mixture (unpublished observation).

Finally, it should be noted that if a DNA probe with a protruding 5'-^{32}P-terminus is incompletely denatured, its labeled terminus may hybridize to the RNA and, after dilution of the formamide, partial DNA renaturation may occur. Since this will lead to the appearance of a long, protected ^{32}P-fragment only if RNA complementary to the probe is present, one may draw erroneous conclusions regarding the length of the RNA. In the case of a DNA probe with a flush 5'-^{32}P end, incomplete denaturation may give rise to S_1-resistant duplexes even in the absence of complementary RNA.

(c) The 5' terminus of the 15 S precursor of mouse β-globin mRNA.

The 15 S precursor RNA of β-globin mRNA has a cap structure indistinguishable from that of the mature mRNA (18). We have now shown that the 5' termini of both species map identically on the genomic DNA; unless an intron exists in the immediate vicinity of the 5' end, one may conclude that the precursor is converted to the mature RNA without incurring any shortening at the 5' terminus.

Does this 5' terminus represent the initiation site of transcription? We have no evidence for a precursor anteceding the 15 S RNA, which would initiate further upstream (18 and unpublished results by P. Curtis); the existence of a 27 S precursor of β-globin mRNA has, however, been claimed (2). Ziff and Evans (30) have found that in the case of adenovirus the 5' end of the late mRNA coincides with the initiation site of transcription. Konkel et al. (13) have shown that, of twelve bases preceding and overlapping the cap nucleotide, ten are identical in adenovirus and mouse β-globin. Moreover, the nucleotide corresponding to the 5' end of a mRNA is preceded, about 23 nucleotides upstream, by a

sequence of 8 nucleotides which is similar for a variety of genes including mouse β-globin, and could represent the promotor site (D. Hogness, personal communication). In view of the mounting evidence that the 5' terminus of a mRNA represents the primary transcription start, we would expect a longer precursor, if it existed, to extend beyond the 3' end rather than the 5' end of the mature mRNA.

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