Translation of globin RNA spliced *in vitro*

Abraham K. ABRAHAM,* Gary BURNS and Mehmet SIMSEK

Biochemistry Department, Faculty of Medicine, Kuwait University, P.O. Box 24923, 13110 Safat, Kuwait

Pre-mRNA (precursor mRNA) enriched for globin-gene transcription products was prepared from murine erythroleukaemia cells induced to differentiate with dimethyl sulphoxide. The pre-mRNA was prepared from nuclear RNA by oligo(dT)–cellulose chromatography followed by sucrose-gradient centrifugation. The pre-mRNA thus obtained was utilized as the substrate for a splicing assay based on sequential splicing and translation reactions. Small nuclear ribonucleoprotein particles, which were shown to inhibit translation, were removed by an intermediate high-speed centrifugation step. The assay developed is based on a three-step procedure: (1) incubation of pre-mRNA with HeLa-cell nuclear extract in the presence of ATP; (2) incorporation of ^3H -labelled amino acid into proteins by wheat-germ extract by using RNA from step 1; and (3) immunoprecipitation of globin chains with rabbit anti-(mouse globin) antibody. No incorporation of label into immune complexes was observed in the absence of HeLa-cell extract or ATP. Preincubation with these components was unnecessary for the incorporation of label into immune complexes when mature cytoplasmic RNA was used as a template for protein synthesis. The assay was further validated by fluorography after polyacrylamide-gel electrophoresis, when the only band detectable corresponded to globin chains. These results demonstrate, for the first time, translational activity of transcripts spliced *in vitro*. The current assay can be utilized with a few micrograms of pre-mRNA and may have potential for further characterization of the splicing system.

INTRODUCTION

Primary transcripts of most eukaryotic genes contain one or more intervening sequences that are subsequently removed by RNA splicing. Attempts to investigate the splicing events further have been facilitated by the development of procedures *in vitro* to assay splicing activity. Accurate splicing *in vitro* of exogenous substrate RNA synthesized from cloned genes has been demonstrated by several groups (Hernandez & Keller, 1983; Padgett *et al.*, 1983; Krainer *et al.*, 1984). In these experiments, the splicing activity has been demonstrated by size determination and sequence analysis of the precursor and product RNA molecules.

If studies of this type are to be useful in elucidating the nature of the splicing system, it must be demonstrated that the messenger spliced *in vitro* meets one further criterion, i.e. that it can serve as a template for synthesis of the appropriate protein.

Although the amount of mRNA produced as described above is sufficient for size and sequence analysis because of its high specific radioactivity, the ability of this system to provide adequate quantities of capped and full-sized mRNA for protein synthesis *in vitro* has not been exploited. Attempts to utilize this type of spliced mRNA in cell-free protein-synthesizing systems are fraught with difficulties, some insurmountable. Firstly, the transcription products of cloned genes and their spliced derivatives usually contain 5' and 3' flanking sequences not present in authentic mRNAs. Secondly, the splicing mixture contains small nuclear RNA species that inhibit translational systems *in vitro* (Rao *et al.*, 1977).

We have overcome these problems by utilizing nuclear RNA produced *in vivo* and enriched for α - and β -globin-

gene transcription products in a sequential splicing/translational system *in vitro*. In the present study we report the use of such a system in the first demonstration that splicing systems *in vitro* produce a translationally active product.

EXPERIMENTAL

Materials

L-[2,3,4,5,6- ^3H]Phenylalanine (TRK-648), L-[4,5- ^3H]leucine (TRK-510) and [α - ^{32}P]dATP were obtained from Amersham International, Amersham, Bucks., U.K. Plasmid pJW 101 was obtained from the American Type Culture Collection (ATCC no. 57601). DNA polymerase 1 and DNAase 1 mixture for nick translation were obtained from Bethesda Research Laboratories, Gaithersburg, MD, U.S.A. Unless otherwise stated, all fine chemicals used were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. All other chemicals were of standard analytical grade.

Cell line

Inducible mouse erythroleukaemia cells (clone 707 B10/1) were obtained from Dr. P. R. Harrison (Beatson Institute, Glasgow, U.K.) Three subclones of the original cell lines were provided by Dr. M. J. Clements (St. George's Hospital Medical School, London, U.K.). The cells were grown in Ham's F12 medium with 10% (v/v) foetal-calf serum (Gibco). The medium was supplemented with 100 units of penicillin/ml and 100 μg of streptomycin/ml. The cell density was kept at 1×10^5 – 2×10^6 cells/ml by adding fresh medium every third or fourth day. To induce differentiation, exponentially growing cultures at a cell density of 1×10^5 cells/ml were

Abbreviation used: pre-mRNA, precursor mRNA.

* To whom correspondence should be addressed.

diluted into growth medium supplemented with 1.5% (v/v) dimethyl sulphoxide. The proportion of haemoglobin-containing cells was determined by scoring benzidine-positive cells as described previously by Benoff & Skoultchi (1977). Haemoglobin content after induction was measured spectrophotometrically in the cell lysate (McClintock & Papaconstantinou, 1974).

Isolation of nuclear and cytoplasmic RNA

Cells were washed with Dulbecco's phosphate-buffered saline without Ca^{2+} and suspended in lysis buffer [140 mM-NaCl, 15 mM- MgCl_2 , 10 mM-Tris/HCl, pH 7.6, plus 50 units of RNasin (RNAase inhibitor; Boehringer)/ml] at 1×10^8 cells/ml. They were lysed with Triton X-100 (final concn. 0.3%, v/v) and the nuclei were pelleted through 1 vol. of 0.6 M-sucrose in lysis buffer by centrifugation at 500 *g* for 10 min in a Sorvall SS-34 rotor at 4 °C. Pelleted nuclei were lysed in 1 vol. of lysis buffer by adding SDS (final concn. 0.5%). SDS was also added to the supernatant fraction to the same final concentration. Guanidinium isothiocyanate was added to both fractions to a final concentration of 4 M, and RNA was extracted by the guanidinium isothiocyanate/hot-phenol method (Maniatis *et al.*, 1982).

Preparation of polyadenylated RNA enriched for globin-mRNA precursors

Polyadenylated RNA was separated from the nuclear fraction by oligo(dT)-cellulose chromatography as described by Abraham & Eikhom (1975) and subjected to sucrose-gradient centrifugation (Abraham *et al.*, 1976) to obtain RNA in the size ranges of 8–10 S and 12–18 S. The latter RNA fraction was used as substrate for the splicing reaction.

Preparation of mRNA

Total polyadenylated RNA prepared from the cytosolic RNA fraction from induced cells was further purified by sucrose-density gradient centrifugation to obtain RNA in the size range of 8–10 S. This fraction served as mRNA for translation assay *in vitro*.

Electrophoresis of RNA under denaturing conditions

Electrophoresis of RNA was carried out on a 1.4%-agarose gel after glyoxylation of RNA as described by McMaster & Carmichael (1977). For detection of RNA after separation, the gel was stained with ethidium bromide. For cDNA hybridization, the glyoxylated RNA was transferred immediately after electrophoresis to a nitrocellulose filter (Thomas, 1980).

Preparation of labelled probe

The plasmid containing α -globin cDNA insert pJW 101 was labelled by a nick-translation procedure (Rigby *et al.*, 1977). The reaction mixture (50 μ l) contained 50 mM-Tris/HCl, pH 7.8, 5 mM- MgCl_2 , 10 mM-2-mercaptoethanol, 20 μ M each of dGTP, dCTP and dTTP, and 300 μ Ci of [α - ^{32}P]dATP (sp. radioactivity 1000 Ci/mmol), 1 μ g of pJW 101 and 10 μ g of bovine serum albumin/ml. After addition of enzyme solution (2 units of DNA polymerase I and 200 pg of DNAase I), the mixture was incubated at 15 °C for 90 min. The reaction was stopped by adding EDTA (final concn. 30 mM). The reaction mixture was passed through a Sephadex G-50 column (1 cm $\times$ 15 cm), and the nick-translated DNA in the void volume was collected.

Approx. 55–60% of the input radioactivity (165–180 μ Ci) was recovered as DNA.

Hybridization of Northern blot with labelled cDNA probe

Both prehybridization and hybridization were performed at 42 °C in sealed plastic bags as described by Simsek *et al.* (1982), by using 50 μ l of hybridization solution/cm² of nitrocellulose filter. Prehybridization was carried out for 2 h, followed by overnight hybridization with approx. 40 μ Ci of labelled probe. The filters were washed for 30 min with 250 ml of each of the following solutions, all containing 0.1% SDS: (1) 2 $\times$ SSC (SSC = 0.15 M-NaCl/0.015 M-sodium citrate) at 25 °C; (2) 1 $\times$ SSC at 42 °C; (3) 0.5 $\times$ SSC at 25 °C; and (4) 0.1 $\times$ SSC at 42 °C. After drying, the filters were exposed to Kodak X-Omat RP film for 1 day at –80 °C and processed.

Splicing system

Soluble nuclear extracts were prepared from HeLa cells as described by Dignam *et al.* (1983). Splicing reactions *in vitro* were carried out in 100 μ l volume at 30 °C for 2 h and contained 60 μ l of nuclear extract (288 μ g of protein) 3.2 mM- MgCl_2 , 500 μ M-ATP, 10 mM-phosphocreatine, 62 mM-KCl, 1 mM-dithiothreitol, 100 μ M-EDTA and 14 μ g of precursor RNA. When indicated, at the end of the reaction samples were centrifuged for 20 min at 148000 *g* in a Beckman Airfuge A-100/18 rotor at 20 °C. The supernatant was collected and used immediately for the translation assay.

mRNA-dependent translational system

Extracts of raw wheat germ (Niblack, Rochester, NY, U.S.A.) were prepared as described by Roberts & Paterson (1973). The standard assay system contained, per 100 μ l: 20 mM-Hepes, pH 7.6, 70 mM-KCl, 3.2 mM- MgCl_2 , 100 μ M-spermidine, 1 mM-dithiothreitol, 1 mM-ATP, 200 μ M-GTP, 10 mM-phosphocreatine, 50 units of creatine kinase, 10 μ M each of 18 non-radioactive amino acids, 1 μ M each (5 μ Ci) of [^3H]leucine and [^3H]phenylalanine, 4 A_{260} units of wheat-germ extract and 50 μ l of high-speed supernatant containing the spliced RNA or appropriate RNA as indicated. Samples were incubated for 40 min at 30 °C.

Antibody precipitation

Samples for immunoprecipitation (50 μ l) were diluted with 200 μ l of phosphate-buffered saline (140 mM-NaCl/15 mM-sodium phosphate containing 0.1% Triton X-100). Then 100 μ l of rabbit anti-(mouse haemoglobin) serum (Cappel; Cooper Biomedical, Malvern, PA, U.S.A.) diluted 1:10 with phosphate-buffered saline was added and the mixture incubated at 22 °C. After 2 h, 100 μ l of Protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) in phosphate-buffered saline was added and incubated at 4 °C overnight. The bound material was collected by centrifugation at 500 *g* for 5 min at 4 °C, and washed three times with 0.5% Triton X-100 in phosphate-buffered saline.

For radioactivity measurements, the beads were suspended in 1 ml of phosphate-buffered saline and filtered through a glass-fibre filter. The filter was washed with 7 $\times$ 5 ml of 5% trichloroacetic acid and then with 2 $\times$ 5 ml of ethanol. Radioactivity was measured in an LKB scintillation counter. For electrophoresis, the

immunoprecipitate was solubilized by heating at 90 °C for 5 min in sample buffer (50 mM-Tris/HCl, pH 6.8, 2 % SDS, 1 % 2-mercaptoethanol, 20 % glycerol and 0.002 % Bromophenol Blue). The clear supernatant after 5 min centrifugation at 10000 *g* (r_{av} . 5.4 cm) was loaded on to the polyacrylamide gels.

Polyacrylamide-gel electrophoresis and fluorography

Polyacrylamide-gel electrophoresis in the presence of SDS was carried out in 0.7 mm-thick 14 cm × 28 cm gels consisting of 14.5 % acrylamide and 5 % bisacrylamide in the Laemmli (1970) system at 10 V/cm for 16 h. The gels were processed for fluorography (Bonner & Laskey, 1974). Dried gels were exposed to Kodak SB film for 1–2 weeks in a Kodak X-Omatic cassette with intensifying screens.

RESULTS AND DISCUSSION

In the past, splicing experiments were conducted with substrate RNA synthesized *in vitro* from a restriction fragment containing the gene in question and a suitable promoter. As noted above, this approach has several advantages in the type of experiments for which they were designed (Hernandez & Keller, 1983; Padgett *et al.*, 1983; Krainer *et al.*, 1984). However, there is a definite disadvantage to this approach if the mRNA produced is to be tested and compared with mRNA produced *in vivo* for its biological activity. As mentioned above the spliced product of substrate RNA produced *in vitro* contains 5' and 3' flanking sequences that are absent from authentic mRNAs.

Our strategy therefore was to use authentic pre-mRNA. Low abundance and sequence homology to corresponding mRNAs make it difficult to purify a specific pre-mRNA even on a cDNA column. However, by choosing a cell line that can be induced to produce a large amount of a specific protein, it is possible to increase the concentration of the corresponding pre-mRNA in the nuclear fraction. Murine erythroleukaemia cells induced to differentiate with dimethyl sulphoxide produce large quantities of haemoglobin (Friend *et al.*, 1971). Since haemoglobin production varies considerably, depending on the cell line, and even in subclones of a given cell line (Marks & Rifkind, 1978), all experiments were carried out with subclone I of clone 707 B10/1, which produced maximum haemoglobin under our conditions for erythroid differentiation (results not shown).

The rate of globin transcription in erythroleukaemia cells after induction by dimethyl sulphoxide, which corresponds to the rate of appearance of mature mRNA transcript in the cytoplasm, increased significantly on day 4 after addition of dimethyl sulphoxide (Ganguly & Skoultschi, 1985). Therefore nuclear RNA was extracted from cells 96 h after addition of dimethyl sulphoxide, as described in the Experimental section. For further purification of this RNA fraction, our aim was to make sure that it was completely free of mature globin mRNAs. Since absolute purity of globin pre-mRNAs was not essential for our experiments, the following procedure was adopted. Polyadenylated RNA was first separated from total nuclear RNA by oligo(dT)–cellulose chromatography. The large size difference between globin pre-mRNAs and mRNAs was then utilized to

Table 1. Ability of polyadenylated nuclear RNA fractions to synthesize globin chains

Nuclear polyadenylated RNA (210 µg) in 200 µl of gradient buffer (100 mM-Tris/HCl, pH 7.4, 1 mM-EDTA, 0.5 % SDS) was treated at 50 °C for 2 min, cooled, layered on top of a 15–30 % sucrose gradient in the above buffer and centrifuged in a Beckman SW 41 Ti rotor. The rotor was operated for 9 h at 20 °C and 197000 *g* (r_{av} . 11.02 cm). Fractions corresponding to 8–10 S and 12–18 S RNA were collected and purified by repeated ethanol precipitation. Markers were 5.8 S, 18 S and 28 S rRNA and rabbit reticulocyte globin mRNA (9 S). The purified RNA fractions dissolved in water were tested for their ability to incorporate radioactive amino acids into proteins as described in the Experimental section by using 5 µg of nuclear polyadenylated RNA. Radioactivity in globin chains was determined after immunoprecipitation as described above.

Template RNA	Amino acid incorporated (d.p.m./assay)	
	Total	Immunoprecipitate
Fraction 1 (8–10S)	113000	61500
Fraction 2 (12–18 S)	92900	—

obtain a complete separation in a single sucrose-gradient step.

The ability of the two fractions of polyadenylated nuclear RNA separated on a sucrose gradient to stimulate amino acid incorporation into proteins and immunoprecipitable globin chains is shown in Table 1. Although there was stimulation of amino acid incorporation by both RNA fractions, only RNA fraction 1 (8–10 S) was able to stimulate amino acid incorporation into immunoprecipitable proteins. All splicing experiments were conducted with the RNA fraction 2 (12–18 S) that did not contain mature globin mRNAs.

It could, however, be argued that our nuclear polyadenylated RNA (fraction 2) still contained some aggregated globin mRNA, since both oligo(dT)–cellulose chromatography and sucrose-gradient centrifugation were performed under non-denaturing conditions. Therefore we analysed this hnRNA fraction after electrophoretic separation under fully denaturing conditions (McMaster & Carmichael, 1977), followed by Northern blotting and hybridization with globin cDNA probe (Simsek *et al.*, 1982). No hybridization of the probe to rRNA was detected (Fig. 1, lane 1). Lane 2 shows the hybridization of the probe to reticulocyte 9 S mRNA and lane 3 to the nuclear polyadenylated RNA (fraction 2). Aggregation of mRNA was not observed (lane 2). Furthermore, no hybridization in the mRNA region was detected in the fractionated pre-mRNA sample (lane 3). Altogether, these results clearly indicate that the substrate RNA used in the splicing reaction did not contain processed 9 S globin mRNA.

Our next objective was to develop an assay system for testing the translational activity of this RNA fraction after splicing. To overcome the problems associated with the purification of small amounts of mRNA, we attempted to use the splicing reaction mixture as such in a sequential splicing/translational assay. Splicing was

Fig. 1. Autoradiogram of RNA samples separated by electrophoresis under denaturing conditions, and hybridized to ^{32}P -labelled α -globin cDNA

Glyoxylated RNA samples were separated on a horizontal 1.4%-agarose gel (McMaster & Carmichael, 1977) for 90 min at 5 V/cm. Lanes 1, 2 and 3 were blotted on to a nitrocellulose filter (Thomas, 1980), and the filter was baked and hybridized to ^{32}P -labelled α -globin cDNA probe as described in the Experimental section. Lane 1, 5 μg of rRNA; lane 2, 2 μg of globin mRNA; lane 3, 7 μg of pre-mRNA. Positions of molecular size markers, DNA (right; b, bases) and rRNA (left), are indicated by arrows. These positions were determined by ethidium bromide staining.

carried out with HeLa-cell nuclear extracts prepared as described by Dignam *et al.* (1983) and assayed as described by Krainer *et al.* (1984). The feasibility of this approach was tested by studying the effect of HeLa-cell nuclear extract alone and the total splicing reaction mixture on the translation of purified globin mRNAs in wheat-germ extracts. In these experiments the concentrations of constituents that are common to both systems, such as MgCl_2 , KCl etc., were adjusted to the optimum concentrations for translation (see the Experimental section). Both the splicing mixture and HeLa-cell nuclear extract showed a significant inhibitory effect on protein synthesis (Table 2). The extent of inhibition was similar in both cases, suggesting that nuclear extract alone is responsible for the observed inhibition. Since HeLa-cell nuclear extract prepared as described by Dignam *et al.* (1983) has been successfully used by several groups for accurate splicing of several pre-mRNAs, it is unlikely that the observed inhibition is caused by the breakdown of mRNA. These extracts contain significant quantities of ribonucleoprotein particles (Grabowski & Sharp, 1986), which contain small nuclear RNAs. It therefore seemed most likely that the ribonucleoprotein particles were responsible for the inhibition of translation. In fact, inhibition of translation *in vitro* by small nuclear and cytoplasmic RNAs has been reported previously (Rao *et al.*, 1977; Bester *et al.*, 1975).

HeLa-cell nuclear extract from which most of the ribonucleoprotein particles had been removed by high-speed centrifugation at 148 000 g for 20 min in a Beckman Airfuge A 30/18 rotor inhibited translation *in vitro* to a much lesser extent (Table 2). Thus the inhibitory effect of

Table 2. Effect of HeLa-cell nuclear extract on protein synthesis in wheat-germ extract

Protein synthesis was assayed by measuring the incorporation of [^3H]phenylalanine and [^3H]leucine in the standard wheat-germ system as described in the Experimental section by using 1 μg of rabbit globin mRNA. In samples containing splicing mixture of HeLa-cell nuclear extract, the concentrations of ATP, phosphocreatine, KCl , MgCl_2 and dithiothreitol were adjusted to the optimum for the wheat-germ system (see the Experimental section). No pre-mRNA was added to the splicing mixture or HeLa-cell extract. High-speed supernatant of splicing mixture was prepared by centrifugation at 148 000 g for 20 min in a Beckman Airfuge A30/18 rotor.

Additions	Radioactivity incorporated (d.p.m./assay)	Inhibition (%)
None	78 600	
Nuclear extract (144 μg)	30 400	61
Splicing mixture (50 μl) containing 144 μg of nuclear extract	29 800	62
Splicing mixture (50 μl) after high-speed centrifugation	62 800	20

the splicing reaction mixture could be decreased significantly by this simple operation, making it possible to assay the template activity of RNA spliced *in vitro* in a sequential splicing/translational system.

The template activity of globin mRNAs spliced *in vitro* assayed directly in this manner is shown in Table 3. No globin chain synthesis was detected with the substrate RNA used for the splicing reaction. Also, the splicing reaction carried out in the absence of ATP showed no significant globin chain synthesis, whereas splicing reactions carried out under optimal conditions (Krainer *et al.*, 1984) produced mRNA capable of stimulating amino acid incorporation into immunoprecipitable globin chains (Table 3). Significant incorporation of amino acids, even when the splicing was carried out in the absence of ATP, into proteins that are not immunoprecipitable was caused by the presence of non-globin mRNAs already present in the 12–18 S RNA fraction (Table 1). During the long incubation period of the splicing reaction, the translational activity of this RNA is somewhat decreased.

By comparison with globin chains synthesized by cytoplasmic globin mRNA (Table 3), it can be inferred that the amount of globin mRNAs produced in the splicing reaction was roughly equivalent to 900 ng. Although the efficiency of splicing may not seem impressive at first sight, i.e. less than 13 % of input RNA, one has to bear in mind that the substrate RNA used here was not pure globin pre-mRNA. From the ethidium bromide staining pattern of the substrate RNA (results not shown), it could also be seen that a significant amount of material moved in the region of 18 S RNA and did not hybridize with the probe, indicating a

Table 3. Globin-chain synthesis by RNA spliced *in vitro*

Protein synthesis was carried out in wheat-germ extracts as described in the Experimental section. Samples of RNA spliced *in vitro* were added to the translational system as 50 μ l portions of high-speed supernatant of the splicing reaction mixture prepared as described in the Experimental section. Translational system containing non-spliced nuclear RNA and cytoplasmic mRNA also contained 50 μ l of high-speed supernatant prepared from a splicing reaction mixture to which no substrate RNA was added. The concentrations of cytoplasmic mRNA and nuclear polyadenylated RNA were 2 and 7 μ g respectively. Total and immunoprecipitable radioactivity were measured as described above.

RNA source	Splicing conditions	Radioactivity incorporated (d.p.m./assay)	
		Total	Immuno-precipitate
Polyadenylated nuclear RNA (12–18 S)	Complete	79 300	43 800
	– ATP	33 000	90
Polyadenylated nuclear RNA (12–18 S)	Not spliced	62 000	35
Cytoplasmic mRNA (8–10 S)	–	144 000	109 000

splicing efficiency much greater than the value indicated above. A fluorogram of the immunoprecipitate after SDS/polyacrylamide-gel electrophoresis is shown in Fig. 2. The only major radioactive band detectable in the immunoprecipitate corresponded to that of globin chains (lane 1). The position of this band was identical with the position of the translation product of cytoplasmic globin mRNAs (lane 3).

The procedure described here demonstrates the formation of biologically active mRNA in a splicing reaction *in vitro*. Sequential assay of splicing and translation avoids the purification and possible loss of mRNA after the splicing reaction. Using tritiated amino acids, we were able to detect the formation of nanogram quantities of mRNA. It should be possible to improve the sensitivity of this system further by using [35 S]methionine. Thus translational assays of splicing reactions may permit a more quantitative approach to the characterization of the splicing system.

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Fig. 2. Fluorogram of immunoprecipitated proteins after SDS/polyacrylamide-gel electrophoresis

Washed immunoprecipitate was dissolved in denaturing buffer heated for 5 min at 90 °C and subjected to electrophoretic separation as described in the Experimental section. The translational system contained RNA splicing mixture (lanes 1 and 2) and cytoplasmic mRNA (lane 3). Immunoprecipitate corresponding to 20 000 d.p.m. was applied to the gel (lanes 1 and 3). Lane 2 contained no ATP during the splicing reaction, and the total immunoprecipitate (90 d.p.m.) was applied to the gel.

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