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Introduction and expression of a rabbit β-globin gene in mouse fibroblasts

(DNA-mediated gene transfer/cotransformation/intervening sequences/gene regulation)

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ABSTRACT **The cloned chromosomal rabbit β-globin gene has been introduced into mouse fibroblasts by DNA-mediated gene transfer (transformation). In this report, we examine the expression of the rabbit gene in six independent transformants that contain from 1 to 20 copies of the cloned globin gene. Rabbit globin transcripts were detected in two of these transformants at steady-state concentrations of 5 and 2 copies per cell. The globin transcripts from one cell line are polyadenylylated and migrate as 9S RNA on methylmercury gels. These transcripts reflect correct processing of the two intervening sequences but lack 48 ± 5 nucleotides present at the 5′ terminus of rabbit erythrocyte globin mRNA.**

Cellular genes coding for selectable biochemical functions can be stably introduced into cultured mammalian cells by DNA-mediated gene transfer (transformation) (1, 2). Biochemical transformants are readily identified by the stable expression of a gene coding for a selectable marker. These transformants represent a subpopulation of competent cells that integrate other physically unlinked genes for which no selective criteria exist (3). In this manner, we have used a viral thymidine kinase (tk) gene as a selectable marker to isolate mouse cell lines that are stably transformed with the tk gene along with bacteriophage φX174, plasmid pBR322, or the cloned chromosomal rabbit β-globin gene sequences (3).

Cotransformed mouse fibroblasts containing the rabbit β-globin gene provide an opportunity to study the expression and subsequent processing of these sequences in a heterologous host. In this report, we demonstrate the expression of the transformed rabbit β-globin gene generating a discrete polyadenylylated 9S species of globin RNA. This RNA results from correct processing of both intervening sequences, but lacks approximately 48 nucleotides present at the 5′ terminus of mature rabbit β-globin mRNA.

MATERIALS AND METHODS

Cell Culture. Murine Ltk$^-$ aprt$^-$ cells are adenine phosphoribosyltransferase-negative derivatives of Ltk$^-$ clone 1D cells (4) that were originally isolated and characterized by R. Hughes and P. Plagemann. Cells were maintained in growth medium and prepared for transformation as described (5).

Transformation and Selection. The transformation protocol, selection for tk$^+$ transformants, and maintenance of transformant cell lines were as described (5).

DNA Isolation. DNA was extracted from cultured L cells as described (5). Recombinant phage containing the rabbit β-globin gene in the λ phage vector Charon 4A were grown and purified, and DNA was isolated as described (6). The herpes virus DNA fragment containing the tk gene was purified from total DNA of herpes simplex virus strain F (7). Intact herpes virus DNA was digested with the restriction endonuclease *Kpn* I and fractionated by agarose gel electrophoresis, and the 5.1-kilobase pair (kbp) fragment containing the tk gene was extracted from the gel as described (8).

RNA Isolation. Total RNA was isolated from logarithmic-phase cultures of transformed L cells by successive extractions with phenol at pH 5.1, phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol), and chloroform/isoamyl alcohol (24:1, vol/vol). After ethanol precipitation, the RNA was digested with DNase (9) and precipitated with ethanol. Nuclear and cytoplasmic fractions were isolated as described (5) and RNAs were extracted as described above. Cytoplasmic polyadenylylated RNA was isolated by oligo(dT)-cellulose chromatography (10).

cDNA Synthesis. Rabbit and mouse cDNAs were prepared by using avian myeloblastosis virus reverse transcriptase (RNA-dependent DNA polymerase) (obtained from J. W. Beard), as described (11).

DNA Filter Hybridizations. Cellular DNA was digested with restriction endonucleases, electrophoresed on agarose slab gels, transferred to nitrocellulose filter sheets, and hybridized with ^{32}P-labeled DNA probes as described by Wigler *et al.* (5).

Solution Hybridizations. ^{32}P-Labeled globin cDNAs (specific activities of 2–9 × 10^8 cpm/μg) were hybridized with excess RNA in 0.4 M NaCl/25 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.5/5 mM EDTA at 75°C. Incubation times did not exceed 70 hr. R_0ts were calculated as moles of RNA nucleotides per liter times time in seconds. The fraction of cDNA rendered resistant to the single-strand nuclease S1 in hybridization was determined as described (10).

RNA Filter Hybridizations. RNA was electrophoresed through 1% agarose slab gels (17 × 20 × 0.4 cm) containing 5 mM methylmercury hydroxide as described by Bailey and Davidson (12). The concentration of RNA in each slot was 0.5 μg/μl. Electrophoresis was at 110 V for 12 hr at room temperature.

RNA was transferred from the gel to diazotized cellulose paper as described by Alwine *et al.* (13) by using pH 4.0 citrate transfer buffer. After transfer, the RNA filter was incubated for 1 hr with transfer buffer containing carrier RNA at 500 μg/ml. The RNA on the filters was hybridized with cloned DNA probe at 50 ng/ml labeled by ^{32}P nick translation (14) to specific activities of 2–8 × 10^8 cpm/μg. Reaction volumes were

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Abbreviations: tk, thymidine kinase; kbp, kilobase pairs; Pipes, 1,4-piperazinediethanesulfonic acid; R_0t, product of RNA concentration (moles of nucleotide per liter) and incubation time (seconds).

25 μl/cm^2 of filter. Hybridization was in 4× standard saline citrate (0.15 M NaCl/0.015 M sodium citrate)/50% formamide at 57°C for 36–48 hr.

After hybridization, filters were soaked in two changes of 2× standard saline citrate/25 mM sodium phosphate/1.5 mM sodium pyrophosphate/0.1% sodium dodecyl sulfate/5 mM EDTA at 37°C for 30 min with shaking to remove formamide. Successive washes were at 68°C with 1× and 0.1× standard saline citrate containing 5 mM EDTA and 0.1% sodium dodecyl sulfate for 30 min each.

Berk–Sharp Analysis of Rabbit β-Globin RNA in Transformed Mouse L Cells. The hybridizations were carried out in 80% (vol/vol) formamide (Eastman)/0.4 M Pipes, pH 6.5/0.1 mM EDTA/0.4 M NaCl (15, 16) for 18 hr at 51°C for the 1.8 kbp *Hha* I fragment and 49°C for the *Pst* I fragment. The hybrids were treated with S1 nuclease and analyzed by a modification of the procedure described by Berk and Sharp (16).

RESULTS

Transformation of mouse cells with the rabbit β-globin gene

We have performed cotransformation experiments with the chromosomal adult rabbit β-globin gene, using the purified herpes virus tk gene as a biochemical marker. The addition of the tk gene to mutant Ltk$^-$ mouse fibroblasts results in the appearance of stable transformants that can be selected by their ability to grow in hypoxanthine/aminopterin/thymidine (HAT) medium. Cells were cotransformed with a β-globin gene clone designated RβG1, which consists of a 15.5-kbp insert of rabbit DNA carried in the bacteriophage λ cloning vector Charon 4A (Fig. 1*A*) (unpublished data). The purified tk gene was mixed with a 100-fold molar excess of intact recombinant DNA from clone RβG1. This DNA was then exposed to mouse Ltk$^-$ cells under transformation conditions previously described (5). After 2 weeks in selective medium, tk$^+$ transformants were observed at a frequency of one colony per 10^6 cells per 20 pg of tk gene. Clones were picked and grown into mass culture.

We then asked if the tk$^+$ transformants also contain rabbit β-globin sequences. High molecular weight DNA from eight transformants was cleaved with the restriction endonuclease *Kpn* I. The DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters, and these filters were then annealed with nick-translated globin [^{32}P]DNA [blot hybridization (17)]. Cleavage of this recombinant phage with the enzyme *Kpn* I generates a 4.7-kbp fragment that contains the entire adult β-globin gene, along with 1.4 kbp of 5′ flanking information and 2.0 kbp of 3′ flanking information (Fig. 1*A*). This fragment was purified by gel electrophoresis and nick translated to generate a hybridization probe. Blot hybridization experiments showed that the 4.7-kbp *Kpn* I fragment containing the globin gene was present in the DNA of six of the eight tk$^+$ transformants. In three of the clones (Fig. 2, lanes E, F, and H), additional rabbit globin bands were observed, which probably resulted from the loss of at least one of the *Kpn* I sites during transformation. The number of rabbit globin genes integrated in these transformants was variable: some clones contained a single copy of the gene (Fig. 2, lanes J and K), whereas others contained up to 20 copies of the heterologous gene. It should be noted that the β-globin genes of mouse and rabbit are partially homologous. However, we do not observe hybridization of the rabbit β-globin probe to *Kpn*-cleaved mouse DNA, presumably because *Kpn* cleavage of mouse DNA leaves the β-gene cluster in exceedingly high molecular weight fragments not readily detected in these experiments (Fig. 2). These results demonstrate the introduction of the cloned chromosomal rabbit β-globin gene into mouse cells by DNA-mediated gene transfer.

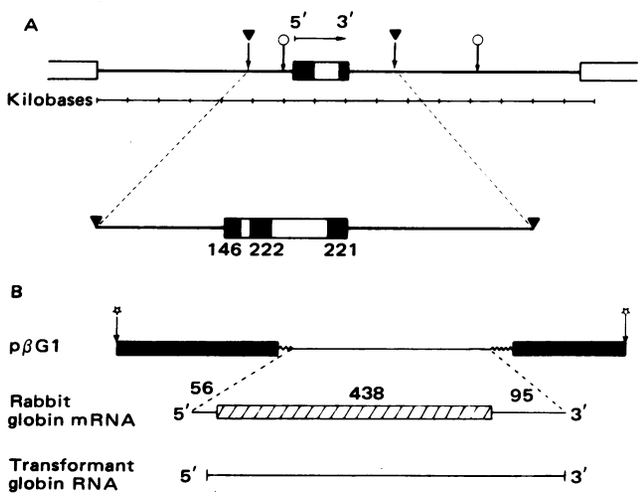

FIG. 1. (*A*) Structure of the rabbit β-globin genomic clone RβG1. The solid box represents the mRNA coding sequence in the adult β-globin gene. The clear regions bounded by coding sequence indicate the intervening sequences within the β-globin gene. The larger 3′ intervening sequence is about 600 base pairs long and the smaller 5′ sequence (shown only in the lower map) is about 125 base pairs long. Restriction sites are indicated by arrows: ▼, *Kpn* I; ○, *Pst* I. (*B*) Structure of the cDNA clone pβG1 and rabbit β-globin mRNA. The *Hha* I restriction fragment of pβG1 is shown. The heavy black lines indicate pMB9 plasmid vector sequence and the thin straight line indicates rabbit mRNA sequence: ☆ *Hha* I sites. The map of rabbit globin mRNA shows the 438-nucleotide translated region bounded by the 5′ 56-nucleotide untranslated region and the 3′ 95-nucleotide untranslated region. The bottom map is of cytoplasmic polyadenylated rabbit globin RNA from transformant cell line 6, which lacks approximately 48 nucleotides of 5′ mRNA sequence (see *Results*).

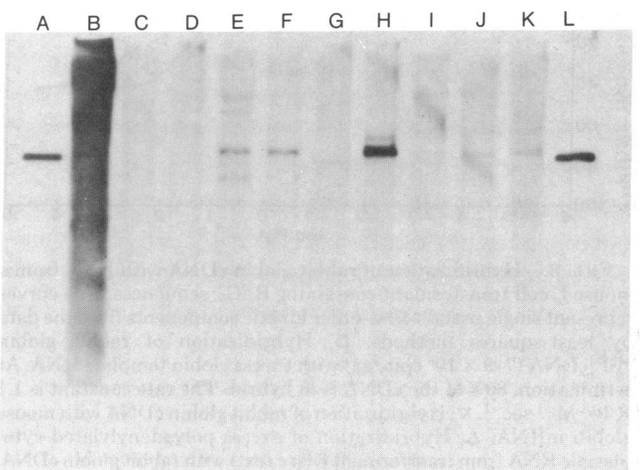

FIG. 2. Rabbit β-globin genes in transformed mouse L cells. High molecular weight DNA from eight independent cotransformant clones was digested with *Kpn* I and electrophoresed on a 0.7% agarose gel. The DNA was denatured *in situ* and transferred to nitrocellulose filters, which were then annealed with a ^{32}P-labeled 4.7-kbp fragment containing the rabbit β-globin gene. Lanes A and L, 50 pg of the 4.7-kbp *Kpn* fragment of RβG1; lane B, 15 μg of rabbit liver DNA digested with *Kpn*; lane C, 15 μg of Ltk$^-$ aprt$^-$ DNA; lanes D–K, 15 μg of DNA from each of eight independently isolated tk$^+$ transformants.

Rabbit β-globin sequences are transcribed in mouse transformants

The cotransformation system we have developed may provide a functional assay for cloned eukaryotic genes if these genes are expressed in the heterologous recipient cell. Six transformed cell clones were therefore analyzed for the presence of rabbit β-globin RNA sequences. In initial experiments we performed solution hybridization reactions to determine the cellular concentration of rabbit globin transcripts in our transformants. A radioactive cDNA copy of purified rabbit α- and β-globin mRNA was annealed with a vast excess of cellular RNA. Because homology exists between the mouse and rabbit globin sequences, it was necessary to determine experimental conditions such that the rabbit globin cDNAs did not form stable hybrids with mouse globin mRNA but did react completely with homologous rabbit sequences. At 75°C in the presence of 0.4 M NaCl, over 80% hybridization was observed with the rabbit globin mRNA, whereas the heterologous reaction with purified mouse globin mRNA did not exceed 10% hybridization. The $R_0t_{1/2}$ of the homologous hybridization reaction was 6×10^{-4}, a value consistent with a complexity of 1250 nucleotides contributed by the α- plus β-globin sequences in our cDNA probe (10).

This rabbit globin cDNA was used as a probe in hybridization reactions with total RNA isolated from six transformed cell lines (Fig. 3 and data not shown). Total RNA from transformed clone 6 (Fig. 2, lane H) protected 44% of the rabbit cDNA at completion, the value expected if only β-gene transcripts were present. This reaction displayed pseudo-first-order kinetics with an $R_0t_{1/2}$ of 2×10^3. A second transformant (Fig. 2, lane E) reacted with an $R_0t_{1/2}$ of 8×10^3 (data not shown). No significant hybridization was observed at $R_0ts \geq 10^4$ with total RNA preparations from the four additional transformants.

We have characterized the RNA from clone 6 in greatest detail. RNA from this transformant was fractionated into nuclear and cytoplasmic populations to determine the intracellular localization of the rabbit globin RNA. The cytoplasmic RNA was further fractionated by oligo(dT)-cellulose chromatography into poly(A)$^+$ and poly(A)$^-$ RNA. Poly(A)$^+$ cytoplasmic RNA from clone 6 hybridizes with the rabbit cDNA with an $R_0t_{1/2}$ of 25. This value is 1/80th the $R_0t_{1/2}$ observed with total cellular RNA, consistent with the observation that poly(A)$^+$ cytoplasmic RNA is 1–2% of the total RNA in a mouse cell. Hybridization is not detectable with either nuclear RNA or cytoplasmic poly(A)$^-$ RNA at R_0t values of 1×10^4 and 2×10^4, respectively. The steady-state concentration of rabbit β-globin RNA present in our transformant can be calculated from the $R_0t_{1/2}$ to be about five copies per cell, with greater than 90% localized in the cytoplasm.

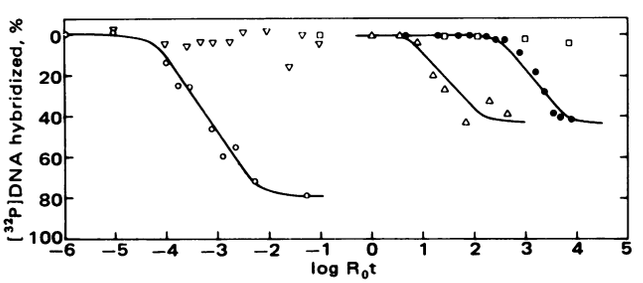

FIG. 3. Hybridization of rabbit globin cDNA with RNA from a mouse L cell transformant containing RβG1 sequences. The curves represent single pseudo-first-order kinetic components fit to the data by least-squares methods. ○, Hybridization of rabbit globin [^{32}P]cDNA ($7–9 \times 10^8$ cpm/μg) with excess globin template RNA. At termination, 80% of the cDNA is in hybrid. The rate constant is 1.1×10^3 M^{-1} sec^{-1}. ▽, Hybridization of rabbit globin cDNA with mouse globin mRNA. △, Hybridization of excess polyadenylylated cytoplasmic RNA from transformant 6 (see text) with rabbit globin cDNA. The rate constant is 2.8×10^{-2} M^{-1} sec^{-1}. The extent of reaction was 43% after normalization for the 70% reactivity of the cDNA at the time of this measurement. ●, Hybridization of excess total cellular RNA from transformant 6 with rabbit globin cDNA. At termination, 43% of the [^{32}P]cDNA was in hybrid. The rate constant is 13.5×10^{-4} M^{-1} sec^{-1}. □, Hybridization of excess nuclear RNA from transformant 6 with rabbit globin cDNA. The S1 resistance of cDNA at zero time has been subtracted from all hybridization values. These background values were 5% and 14% for the cDNA preparations used in this experiment.

Several independent experiments argue that the globin RNA detected derives from transcription of the rabbit DNA sequences present in this transformant: (*i*) cDNA was prepared from purified 9S mouse globin RNA. This cDNA does not hybridize with poly(A)$^+$ RNA from clone 6 at R_0t values at which the reaction with rabbit globin cDNA is complete (Fig. 3). (*ii*) Rabbit globin cDNA does not hybridize with total cellular RNA obtained with tk$^+$ globin$^-$ transformants at R_0t values exceeding 10^4. (*iii*) The hybridization we observe does not result from duplex formation with rabbit globin DNA possibly contaminating the RNA preparations. Rabbit cDNA was annealed with total cellular RNA from clone 6, the reaction product was treated with S1 nuclease, and the duplex was subjected to equilibrium density centrifugation in cesium sulfate under conditions that separate DNA·RNA hybrids from duplex DNA. The S1-resistant cDNA banded at a density of 1.54 g/ml, as expected for DNA·RNA hybrid structures (data not shown). These data, along with the observation that globin RNA is polyadenylylated, demonstrate that the hybridization we observe with RNA preparations does not result from contaminating DNA sequences.

Characterization of rabbit globin transcripts in transformed cells

In rabbit erythroblast nuclei, the β-globin gene sequences are detected as a 14S precursor RNA that reflects transcription of two intervening sequences that are subsequently removed from this molecule to generate a 9S messenger RNA (unpublished results). It was therefore of interest to determine whether the globin transcripts we detected exist as a discrete 9S species, which is likely to reflect appropriate splicing of the rabbit gene transcript by the mouse fibroblast. Cytoplasmic poly(A)-containing RNA from clone 6 was electrophoresed on a methylmercury/agarose gel (12) and transferred to diazotized cellulose paper (13, 18). After transfer, the RNA on the filters was hybridized with DNA from the plasmid pβG1, which contains rabbit β-globin cDNA sequences (19). Using this ^{32}P-labeled probe, we observed a discrete 9S species of RNA in the cytoplasm of the transformant, which comigrated with rabbit globin mRNA isolated from rabbit erythroblasts (Fig. 4). Hybridization to 9S RNA species was not observed in parallel lanes containing either purified mouse 9S globin RNA or poly(A)-containing cytoplasmic RNA from a tk$^+$ transformant containing no rabbit globin genes.

We were unable in these experiments to detect the presence of a 14S precursor in nuclear RNA populations from the transformants. This is not surprising, because the levels expected in nuclear RNA, given the observed cytoplasmic concentration, are likely to be below the limits of detection for this technique. The 5′ and 3′ boundaries of the rabbit globin sequences expressed in transformed fibroblasts along with the internal processing sites can be defined more accurately by hybridizing this RNA with cloned DNAs, followed by S1 nuclease digestion and subsequent gel analysis of the DNA products (16). When

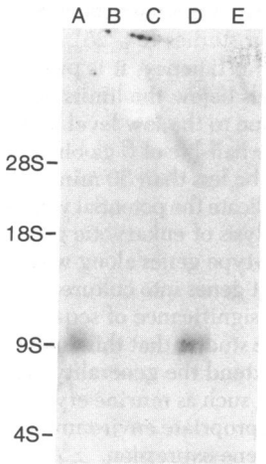

FIG. 4. Sizing of cytoplasmic polyadenylylated rabbit globin transcripts from transformant 6. RNA was electrophoresed in a 1% methylmercury/agarose gel and the RNA was transferred to diazotized cellulose paper. The positions of 28S, 18S, and 4S RNAs on the gel were determined optically after staining with ethidium bromide. The RNA on the filter was hybridized with ^{32}P-labeled plasmid DNA (pβG1) containing the rabbit β-globin cDNA sequence. Lane A, 1 ng of purified 9S polyadenylylated RNA from rabbit reticulocytes, plus 25 μg of carrier chicken oviduct RNA. Lane B, 50 pg of purified 9S polyadenylylated RNA from rabbit reticulocytes, plus 25 μg of carrier chicken oviduct RNA. Lane C, 1 ng of purified 9S polyadenylylated RNA from mouse reticulocytes plus 25 μg of carrier RNA. Lane D, 30 μg of polyadenylylated cytoplasmic RNA from transformant 6. Lane E, 30 μg of cytoplasmic polyadenylylated RNA from a transformant containing no rabbit globin genes.

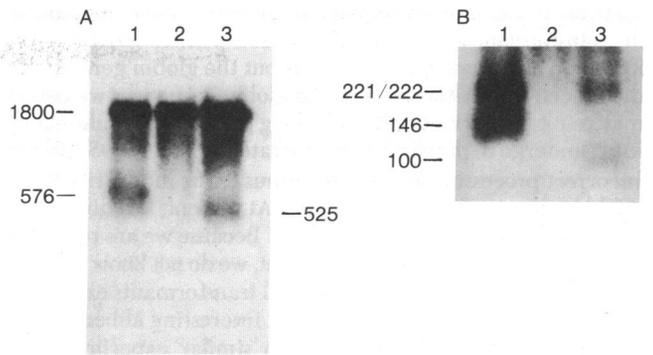

FIG. 5. Characterization of rabbit β-globin RNA in transformed mouse L cells. Numbers of base pairs are given beside the autoradiograms. (*A*) Both total rabbit reticulocyte RNA and poly(A)$^+$ RNA purified from cell line 6 were hybridized to the 1.8-kbp *Hha* I fragment from plasmid pβG1 (Fig. 1*B*) and analyzed as described by Berk and Sharp (16). Lane 1, 0.2 μg of total reticulyocyte RNA was hybridized to 20 ng of the 1.8-kbp *Hha* globin fragment in 5 μl. Lane 2, 18 ng of the 1.8-kbp *Hha* fragment was hybridized in 2.5 μl in the absence of any added RNA. Lane 3, 30 μg of poly(A)$^+$ RNA purified from cell line 6 was hybridized to 75 ng of the 1.8-kbp *Hha* fragment in 10 μl. The 1800-base pair band is the renatured *Hha* fragment. (*B*) Both total rabbit reticulocyte RNA and poly(A)$^+$ RNA purified from cell line 6 were hybridized to a 5.60-kbp *Pst* I fragment containing the genomic copy of the rabbit β-globin gene. The Berk–Sharp analysis was carried out by a procedure to be described elsewhere. Only the bottom half of the autoradiogram is shown and therefore lane-specific background present in lanes 1 and 3, as well as in the RNA$^-$ control (lane 2) is not shown. We believe that this background results from the formation of DNA·DNA duplexes between a small number of nicked *Pst* fragments prior to S1 treatment. Lane 1, 0.35 μg of total rabbit reticulocyte RNA was hybridized to 0.1 μg of the 5.60-kbp *Pst* fragment in 10 μl. Lane 2, 0.12 μg of the *Pst* fragment was hybridized in 10 μl in the absence of any RNA. Lane 3, 30 μg of poly(A)$^+$ RNA purified from cell line 6 was hybridized to 0.12 μg of the 5.60-kbp *Pst* fragment in 10 μl.

β-globin mRNA from rabbit erythroid cells was hybridized with cDNA clone pβG1 (Fig. 1*B*) under appropriate conditions, the entire 576-base pair insert of cDNA was protected from S1 nuclease attack. When this cDNA clone was hybridized with RNA from our transformant, surprisingly, a discrete DNA band was observed at 525 base pairs, but not at 576 base pairs (Fig. 5). These results suggest that, in this transformant, rabbit globin RNA molecules are present that have a deletion in a portion of the globin mRNA sequence at the 5′ or 3′ termini. To distinguish between these possibilities, DNA of the λ clone RβG1, containing the chromosomal rabbit β-globin sequence hybridized with transformed fibroblast RNA. The hybrid formed was treated with S1 nuclease, and the protected DNA fragments were analyzed by alkaline agarose gel electrophoresis and identified by Southern blotting procedures (17). Because the rabbit β-globin gene is interrupted by two intervening sequences, the hybridization of mature rabbit mRNA to RβG1 DNA generates three DNA fragments in this sort of analysis: a 146-base pair fragment spanning the 5′ terminus to the junction of the small intervening sequence, a 222-base pair internal fragment bridging the small and large intervening sequences, and a 221-base pair fragment spanning the 3′ junction of the large intervening sequence to the 3′ terminus of the mRNA molecule (Fig. 1*A*). When transformant RNA was analyzed in this fashion, we observed a 222-base pair fragment and an aberrant fragment of 100 base pairs but no 146-base pair fragment (Fig. 5). Hybridization with a specific 5′ probe showed that the internal 222 base pair fragment was present (data not shown). The sum of the protected lengths equaled the length of the DNA fragment protected by using the cDNA clone. Taken together, these results indicate that although the intervening sequences expressed in transformed mouse fibroblast are removed from the RNA transcripts precisely, the 5′ termini of the cytoplasmic transcripts we observe do not contain about 48 ± 5 nucleotides present in mature 9S RNA of rabbit erythroblasts.

DISCUSSION

In these studies, we have constructed mouse cell lines that contain the rabbit β-globin gene and have analyzed the ability of the mouse fibroblast recipient to transcribe and process this heterologous gene. Solution hybridization experiments in concert with RNA blotting techniques indicate that, in at least one transformed cell line, rabbit globin sequences are expressed in the cytoplasm as a polyadenylylated 9S species. Correct processing of the rabbit β-globin gene has also been observed in tk$^+$ mouse cell transformants in which the globin and tk plasmids have been ligated prior to transformation (20). Similar results have been obtained by using a viral vector to introduce the rabbit globin gene into monkey cells (21, 22). Taken together, these results suggest that nonerythroid cells from heterologous species contain the enzymes necessary to correctly process the intervening sequences of a rabbit gene whose expression usually is restricted to erythroid cells.

The level of expression of rabbit globin sequences in our transformant is low: five copies of globin RNA are present in the cytoplasm of each cell. Our results indicate that the two intervening sequences present in the original globin transcript are processed and removed at loci indistinguishable from those observed in rabbit erythroid cells. Surprisingly, 45 nucleotides present at the 5′ terminus of mature rabbit mRNA are absent

from the β-globin RNA sequence detected in the cytoplasm of the transformant we have examined. It is possible that incorrect initiation of transcription occurs about the globin gene in this mouse cell line. Alternatively, the globin sequences we detect may result from transcription of a long precursor that ultimately must undergo 5′ processing to generate the mature 9S species. Incorrect processing at the 5′ terminus in the mouse fibroblast could be responsible for our results. At present, it is difficult to distinguish among these alternatives. Because we are restricted in our analysis to a single transformant, we do not know whether these observations are common to all transformants expressing the globin gene or reflect a rare but interesting abberation. It should be noted, however, that in similar experiments by Weissmann and his colleagues (20) at least a portion of the rabbit globin RNA molecules transcribed in transformed mouse fibroblasts retain the correct 5′ terminus.

Several alternative explanations can be offered for the expression of globin sequences in transformed fibroblasts. It is possible that constitutive synthesis of globin RNA occurs in cultured fibroblasts (23) at levels five to six orders of magnitude below the level observed in erythroblasts. The introduction of 20 additional globin DNA templates may simply increase this constitutive transcription to the levels observed in our transformant. Alternatively, it is possible that the homologous globin gene is repressed by factors that are partially overcome by a gene dosage effect provided by the introduction of 20 additional globin genes. Finally, normal repression of the globin gene in a fibroblast may depend upon the position of these sequences in the chromosome. At least some of the newly introduced genes are likely to reside at loci distant from the resident mouse globin genes. Some of these ectopic sites may support low level transcription. Our data do not permit us to distinguish among these and other alternatives.

Although the number of rabbit globin genes within a given transformant remains stable for over a hundred generations of culture in hypoxanthine/aminopterin/thymidine (unpublished studies), it has not been possible to prove that these sequences are covalently integrated into recipient cell DNA. In previous studies, however, we have demonstrated that cotransformation of either φX174 or plasmid pBR322 results in the stable integration of these sequences into high molecular nuclear DNA. In the present study, the globin gene represents a small internal segment of the high molecular weight concatenated phage DNA used in the transformation (Fig. 1A). Analysis of integration sites covalently linked to donor DNA is therefore difficult. Preliminary studies using radioactive λ sequences as a probe in DNA blotting experiments indicate that, in some of our cell lines, we have introduced a contiguous stretch of recombinant phage DNA with a minimum length of 50 kbp.

The presence of 9S globin RNA in the cytoplasm of transformants suggests that this RNA may be translated to give rabbit β-globin polypeptide. Attempts to detect this protein in cell lysates using a purified anti-rabbit β-globin antibody (kindly provided by S. Boyer) have thus far been unsuccessful. It is possible that the globin RNAs in our transformant are not translated or are translated with very low efficiency due to the absence of a functional ribosomal binding site. The cytoplasmic globin transcripts in our transformant lack about 48 nucleotides of untranslated 5′ sequence (Fig. 1B), which includes 13 nucleotides known to interact with the 40S ribosomal subunit in nuclease protection studies (24, 25). Even if translation did occur with normal efficiency, it is probable that the protein would exist at levels below the limits of detection of our immunologic assay due to the low level of globin RNA, and the observation that the half-life of β globin in the absence of heme and α globin may be less than 30 min (22).

These studies indicate the potential value of cotransformation systems in the analysis of eukaryotic gene expression. The introduction of wild-type genes along with native and *in vitro*-constructed mutant genes into cultured cells provides an assay for the functional significance of sequence organization. It is obvious from these studies that this analysis will be facilitated by the ability to extend the generality of cotransformation to recipient cell lines, such as murine erythroleukemia cells, that provide a more appropriate environment for the study of heterologous globin gene expression.

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