PRODUCTION OF MOUSE GLOBIN IN HETEROKARYONS OF MOUSE ERYTHROLEUKAEMIA CELLS AND HUMAN FIBROBLASTS

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SUMMARY

In an effort to activate the globin genes of non-erythroid cells, tetraploid murine erythroleukaemia cells (Friend cells) were fused with diploid human amniotic fibroblasts. When the Friend cells were pretreated with dimethylsulphoxide, an average of 27% heterokaryons was observed. These cells stained with benzidine, an indication that they contained haemoglobin. The cells incorporated radioactive amino acids into proteins. Electrophoresis of [3H]leucine-labelled lysates on SDS urea polyacrylamide gels indicated that up to 7% of the newly synthesized protein co-electrophoresed with globin. CM cellulose chromatography demonstrated the presence of mouse but not human globin chains. Hybridization analyses of cytoplasmic RNA also revealed only mouse globin mRNA in the heterokaryons. Although heterokaryons form readily between mouse erythroleukaemia cells and human fibroblasts, and globin synthesis does occur, only the erythroid partner in the fusion system employed here directs globin production.

INTRODUCTION

Heterokaryons formed by the fusion of avian erythroblasts with fibroblasts of other species have been shown to synthesize haemoglobin for up to 3 days following fusion (Alter & Ingram, 1975: Citkowitz, Riggs & Ingram, 1975: Davis & Harris, 1975: Harris, 1970). In these systems, the haemoglobin was avian, and thus was coded for by the erythroblast parent. When hybrid cells were formed from mouse erythroleukaemia cells fused with human or Chinese hamster bone marrow erythroblasts, synthesis of haemoglobin belonging to both parental species was detected (Deisseroth, Barker, Anderson & Nienhuis, 1975). Hybrid cells formed by the fusion of mouse erythroleukaemia cells with heterologous fibroblasts synthesized no haemoglobin at
Synthesis of new proteins has been induced in hybrid cells. Fusion of mouse fibroblasts, which made no albumin, with rat hepatoma cells, which made rat albumin, led to the induction of mouse albumin (Peterson & Weiss, 1972). Similarly, hybrid cells derived from the fusion of human leucocytes with mouse hepatoma cells resulted in the appearance of human albumin (Darlington, Bernhard & Ruddle, 1974). The cell line which was the inducer in these examples was derived from a tumour. In addition, it was hyperdiploid, and thus provided an excessive gene dosage compared to the fibroblast or leucocyte partner in the fusion.

We attempted to duplicate these features with an erythroid system. The erythroblast parent was a tetraploid line derived from mouse erythroleukaemia cells (Friend cells) (Deisseroth, Velez, Burk, Minna, Anderson & Nienhuis, 1976: Friend, Scher, Holland & Sato, 1971). These cells grow in suspension as proerythroblasts, but can be induced to mature to orthochromatic erythroblasts by the addition of dimethyl-sulphoxide (Friend et al. 1971). These tetraploid tumour erythroid cells were fused with diploid human amniotic fibroblasts. A large proportion of heterokaryons was formed, which contained haemoglobin, synthesized globin, and had globin mRNA. Analysis of the globin chains and mRNA in these heterokaryons demonstrated the expression of mouse but not human globin genes.

**MATERIALS AND METHODS**

**Cells**

Human amniotic fibroblasts were kindly provided by Dr. Gail Bruns. The cells were grown in monolayer in Falcon tissue culture dishes (Falcon Plastics, Oxnard, CA). The medium was Dulbecco's modification of Eagle's medium, with 10% heat-inactivated foetal calf serum (Flow Laboratories, Rockville, MD), 100 µg/ml streptomycin, 100 units/ml penicillin, and 0.25 µg/ml amphotericin. The cells were grown in 10% CO₂ in a humidified incubator. Cultures were passed at weekly intervals. All fusion experiments were performed with cells between passages 4 and 14; new stocks were thawed from liquid nitrogen storage as needed. All experiments used cells which derived from a single original amniotic fibroblast culture.

The tetraploid erythroleukaemia cell line (TELC) was established by the fusion of a 6-thioguanine-resistant clone of Friend mouse erythroleukaemia cell line 745 with a BUdR-resistant clone derived from Friend clone 707 (Deisseroth et al. 1976). The TELCs were grown in suspension in Falcon tissue culture flasks in the same medium as above, and passaged twice weekly at 1/20 or 1/40 dilutions. For induction with dimethylsulphoxide (DMSO), the TELCs were plated at 5 x 10⁴ cells/ml in 2% (280 µM) DMSO and cultured for 4 days. This resulted in approximately 60% of the cells staining for haemoglobin with benzidine. The chromosome number was stable at 74 (Moorhead, Nowell, Mellman, Battips & Hungerford, 1960).

**Fusion**

Fusion was performed as previously described (Alter & Ingram, 1975). Fibroblasts were grown in monolayer. Medium was replaced with serum-free medium. One thousand units of β-propiolactone-inactivated Sendai virus (Connaught Laboratories, Toronto, Canada) were added, and the cells incubated at 20°C for 10 min. A 200-fold excess of DMSO-induced or control TELCs was added, and the cultures placed at 37°C for 3 h. The unfused TELCs and virus-containing medium were then replaced with fresh medium with serum.
Protein synthesis

Cells were labelled with [3H]leucine (80 Ci/mM) or [35S]methionine (400 Ci/mM) (New England Nuclear Corp., Boston, MA) in medium in which the radioactive amino acid was limiting. The cultures were harvested and the cells were lysed in 0.5% Nonidet-P40 in 1 mM phosphate, pH 7.4. Nuclei, membranes and debris were removed by centrifugation following the addition of CCl4:toluene (2:1) to the lysates. Evaluation for heterokaryon formation and protein synthesis was done as described previously (Alter & Ingram, 1975). The proportion of total protein synthesis that was globin was determined by electrophoresis on SDS polyacrylamide gels in urea. Globin was eluted from the gels, and chromatographed on CM-52 cellulose (Whatman Biochemicals Ltd., England). The elution buffer was a non-linear gradient of 5-45 mM phosphate in 8 M urea, pH 6.8. Whole globin or isolated globin chains were digested with trypsin and fingerprinted on thin layer (0.1 mm) cellulose plates. [35S]methionine-containing peptides were identified by autoradiography.

Globin mRNA preparation and cDNA synthesis

Total cytoplasmic RNA was prepared from lysates of cultured cells by extraction with SDS and phenol-chloroform-isoamyl alcohol (Aviv & Leder, 1972). Mouse and human reticulocyte globin mRNA were prepared by detergent and phenol extraction of membrane-free hemolysates of peripheral blood cells (Benz & Forget, 1971; Benz, Sverdlow & Forget, 1973, 1975). The globin mRNA was purified from the total reticulocyte RNA by oligo-dT cellulose column chromatography (Aviv & Leder, 1972). Mouse and human globin cDNA were prepared by incubation of the purified reticulocyte mRNA with the RNA-dependent DNA polymerase of avian myeloblastosis virus (kindly provided by Drs D. and J. W. Beard) in the presence of 3H-dCTP (New England Nuclear Corp., 248 Ci/mM) as described previously (Housman, Forget, Skoultchi & Benz, 1973).

Hybridization assay

Saturation hybridization curves were obtained as described previously (Housman et al. 1973). A fixed amount of cDNA (approximately 500 cpm) was incubated with variable amounts of total cytoplasmic RNA in 0.3 M sodium phosphate (pH 6.8) and 0.5% SDS for 40 h at 78 °C. The amount of hybridization was then determined by measurement of the percentage of the cDNA which became resistant to digestion by the S1 nuclease of Aspergillus oryzae as described (Housman et al. 1973).

RESULTS

Heterokaryon formation from the fusion of mouse erythroleukaemia cells with human fibroblasts averaged 10% with uninduced TELCs, and 37% when the TELCs had been grown in 2% DMSO for 4 days prior to fusion. Fig. 1 shows examples of heterokaryons formed from DMSO-induced TELCs and human fibroblasts. The dark nuclei are from the TELCs, and the light nuclei from the fibroblasts. These cells stain light orange with benzidine, which indicates that they contain haemoglobin.

Lysates from heterokaryons which had been labelled with [3H]leucine were analysed by electrophoresis on SDS urea polyacrylamide gels. The region of the gels containing the material of globin molecular weight (16000 Daltons) is presented in Fig. 2. There is no [3H]leucine peak in this region in the electrophoresis from the control fibroblasts (Fig. 2A), while there are peaks in the globin region of the gels from the heterokaryons (Fig. 2B) and the control induced TELCs (Fig. 2C). The radioactivity in the globin region from the fibroblast culture comprised 0.5% of the total protein synthesis.
Fig. 1. Photomicrograph of heterokaryons formed by the fusion of mouse erythro-leukemia cells which had been induced with dimethylsulphoxide, with human fibroblasts. Light nuclei (long arrows) are from fibroblasts, dark nuclei (short arrows) from the erythroblasts. × 1000.

Fig. 2. Electrophoresis on SDS urea polyacrylamide gels of proteins synthesized in vitro. Only the bottoms (globin region) of the gels are shown. Cultures were labelled with [3H]leucine from 3 to 24 h following fusion. A, fibroblasts; B, fusion culture, 71% heterokaryons; C, erythroleukaemia cells which had been previously induced with dimethylsulphoxide. — — —, absorbance of carrier globin, at 280 nm; — — —, cpm × 10^-4 per 2-mm gel segment, calculated per 10^6 cells; ♦, ink marking bromphenol blue dye marker.
Globin synthesis in heterokaryons

Table 1. Globin synthesis in erythroblast × fibroblast heterokaryons 24 h after fusion

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Sample</th>
<th>% hetero-karyons</th>
<th>Net globin as % of total protein</th>
<th>Label interval, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fibroblasts</td>
<td>17</td>
<td>1</td>
<td>3–24</td>
</tr>
<tr>
<td></td>
<td>fusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TELC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fibroblasts</td>
<td>71</td>
<td>0.5</td>
<td>3–24</td>
</tr>
<tr>
<td></td>
<td>fusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TELC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Fibroblasts</td>
<td>38</td>
<td>0.4</td>
<td>20–24</td>
</tr>
<tr>
<td></td>
<td>fusion</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>TELC</td>
<td></td>
<td></td>
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</tr>
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</table>

Cultures were labelled with [3H]leucine.

* cpm in globin region of SDS urea gel – gel background total cpm recovered on gel

Table 2. Time course of globin synthesis in erythroblast × fibroblast heterokaryons

<table>
<thead>
<tr>
<th>Day</th>
<th>Sample</th>
<th>% hetero-karyons</th>
<th>No. of hetero-karyons</th>
<th>% net globin</th>
<th>Globin cpm per 10⁶ cells</th>
<th>Globin cpm per 10⁶ hetero-karyons*</th>
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<tbody>
<tr>
<td>1</td>
<td>Fibroblasts</td>
<td>38</td>
<td>38760</td>
<td>0.4</td>
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<td>2</td>
<td>41,549</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>TELC</td>
<td></td>
<td>23</td>
<td>3401</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fibroblasts</td>
<td>24</td>
<td>8808</td>
<td>0.4</td>
<td>8,168</td>
<td>85,662</td>
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<td></td>
<td>2</td>
<td>82,727</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>TELC</td>
<td></td>
<td>10</td>
<td>3055</td>
<td></td>
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</tr>
<tr>
<td>3</td>
<td>Fibroblasts</td>
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<td>0.6</td>
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</tr>
<tr>
<td></td>
<td>TELC</td>
<td></td>
<td>3</td>
<td>831</td>
<td></td>
<td></td>
</tr>
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</table>

Cultures were labelled with [3H]leucine for 20 h

* Net globin per 10⁶ fusion cells – net globin per 10⁶ fibroblasts x 100 % heterokaryons

(Table 1, experiment 2). The data from 2 similar experiments are also outlined in Table 1. In these experiments, all unfused erythroblasts had been removed from the fusion cultures by extensive washing. The apparent globin synthesis was thus occurring in the monolayer of heterokaryons and unfused fibroblasts, and was not due to any residual erythroblast contamination.

Globin synthesis by these heterokaryons was examined over a period of 3 days (Table 2). The globin synthesis was maximal in the first 2 days, and then declined. Part of the decline was due to overgrowth of the culture by unfused fibroblasts,
although the absolute number of heterokaryons also declined. In addition, globin synthesis per $10^6$ heterokaryons or TELCs also decreased on the third day. At all time points, the radioactivity in globin per $10^6$ heterokaryons was approximately 20 times as much as that in $10^6$ TELCs.

Fig. 3. Radiochromatogram of globin recovered from SDS urea gels. Cultures were labelled with $[3H]$leucine from 3 to 24 h. A, fibroblasts; B, fusion culture, 71% heterokaryons; C, erythroleukaemia cells which had been previously induced with dimethylsulphoxide, $[14C]$leucine, cpm x $10^3$, of carrier human globin; ---, $[H]$leucine, cpm x $10^3$; H, human; M, Mouse.

The globin molecular weight material was further analysed to establish conclusively that it was globin, and to determine the species of its origin. Elution of the material in this region from SDS urea gels led to the elimination of 99% of the fibroblast proteins. CM-cellulose chromatograms are shown in Fig. 3. Human fibroblasts did not synthesize any proteins which co-chromatographed with globin chains (Fig. 3A). The elution profiles from the fusion culture (Fig. 3B) and the TELCs are very similar (Fig. 3C). There is no peak of radioactivity in the area of the human $\gamma$ chain. The next peak, labelled mouse pre-$\beta$, coincided with human $\beta$-chains. The proportion of this peak to mouse $\beta$ radioactivity is similar in the fusion and the TELC column chromatograms, which suggests that a large amount of human $\beta$ chain was not synthesized in the heterokaryons. There is also no radioactivity in the column shown in
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Fig 3B in the area of the human α chain, the last peak to come off the column. Thus, the heterokaryons appear to have synthesized mouse β and α chains, but no human chains. The mouse pre-β and pre-α peaks are artifacts of the system, and have been shown by fingerprinting to contain mouse β and α peptides.

Fig. 4. Specificity of hybridization of globin cDNA probes used for analysis of RNA by hybridization. Saturation hybridization assays were carried out as described in Materials and methods, using a fixed amount of cDNA and varying amounts of total cellular RNA. A, mouse globin cDNA; B, human globin cDNA; •, mouse reticulocyte RNA; ○, human reticulocyte RNA.

Cells were also labelled with [35S]methionine for the first 24 h following fusion. Fingerprints of globin recovered from SDS-urea gels showed the presence of mouse but not human methionine peptides.

The globin mRNA content of the heterokaryons was examined by hybridization with globin cDNA from human and murine sources. Under the experimental conditions used, the hybridization assay specifically detects mouse and human globin mRNA sequences with very little cross-species hybridization (Fig. 4). Mouse reticulocyte RNA hybridizes with human globin cDNA to an extent of only 5% (Fig. 4B), and human reticulocyte RNA hybridizes with the mouse globin cDNA to the extent of approximately 10% (Fig. 4A). On the other hand, plateaus of 65 and 80%
hybridization are obtained in the human-human and mouse-mouse mRNA-cDNA hybridization assays respectively (Fig. 4).

Cytoplasmic RNA from control uninduced TELCs failed to hybridize with the mouse cDNA, but RNA from cells induced to differentiate by DMSO did hybridize with the mouse cDNA. Half-maximum hybridization (Fig. 5A) was achieved at an

![Graph](image_url)

**Fig. 5.** Hybridization of cytoplasmic RNA from TELCs, fibroblasts and fusion cells. Conditions as in Fig. 4. A, erythroleukaemia RNA hybridized with mouse globin cDNA; DMSO-induced TELCs; uninduced TELCs. B, experimental culture RNA: fusion culture RNA hybridized with mouse globin cDNA; fusion culture RNA hybridized with human globin cDNA; fibroblast RNA hybridized with mouse or human globin cDNA.

RNA input approximately 10 times that of the total reticulocyte RNA (Fig. 4A). RNA from the fibroblasts failed to give any hybridization with either mouse or human cDNA, at a maximum total RNA input of 4 μg (Fig. 5B). RNA from the fibroblast × TELC fusion cultures, on the other hand, gave significant hybridization with the mouse cDNA, but there was no significant hybridization with the human cDNA even at an RNA input of 10 μg (Fig. 5B). The cell preparation from which the RNA was extracted for this experiment contained some unfused TELCs, as well as 53% heterokaryons. We cannot therefore state that all of the hybridizable mouse globin mRNA was derived only from the heterokaryons. It is clear, however, that there were
no detectable human globin mRNA sequences in the cytoplasmic RNA of this cell population.

DISCUSSION

Because fibroblasts had been induced to synthesize proteins which they did not previously produce (Darlington et al. 1974: Peterson & Weiss, 1972), and erythroblast x fibroblast heterokaryons did synthesize haemoglobin (Alter & Ingram, 1975; Citkowitz et al. 1975; Davis & Harris, 1975; Harris, 1970), our experiments were designed to maximize the possibility that the haemoglobin in heterokaryons would originate in the fibroblast parent. The erythroblasts were tetraploid, which doubled the gene input from this parent. In addition, the erythroblasts came from a tumour line (erythroleukaemia). The fusion was of 2 mammalian cell types, to avoid any potential problems that may have arisen from crossing species lines.

Heterokaryon formation did occur readily. A maximum of 71% fused cells was observed. Many of the heterokaryons contained multiple erythroblast nuclei. Globin synthesis was examined at various times during the first 3 days following fusion. At the end of 3 days, most of the heterokaryons had disappeared, and globin synthesis had declined. While it is possible that the decrease in heterokaryon number was due to hybridization, it is more likely that the cells had died. Citkowitz et al. (1975) showed that the heterokaryons divided much more slowly than fibroblasts present in the same culture.

The amount of radioactivity in globin per 10⁵ heterokaryons exceeded that amount per 10⁷ TELCs. Davis & Harris (1975) had indicated that there was an apparent stimulation of haemoglobin synthesis in heterokaryons relative to erythroblasts. Alter & Ingram (1975) showed that such an apparent stimulation could be completely explained by the differences in intracellular leucine pool sizes. Thus, more radioactivity in heterokaryon globin is most likely due to a smaller dilution of the added [3H]leucine by intracellular unlabelled leucine.

Our data indicate that the globin synthesized by the heterokaryons was coded for by the mouse erythroblast partner in the fusion, and that human globin was not produced in quantities which could be detected by our assays. The chromatograms showed that no human γ or α chains were produced. While the human β chain did not separate from the mouse pre-β peak, there was no increase in the radioactivity in this area in the globin from the heterokaryons, when compared to that produced by the tetraploid erythroleukaemia cells. Because of the high level of background radioactivity, this column procedure might fail to detect globin chains which comprise less than 5% of the major peak levels. We can only conclude that human globin chain synthesis was not apparent with this assay.

The analysis of globin mRNA by RNA-cDNA hybridization provided a more sensitive method for the evaluation of possible human globin gene transcription. The human globin cDNA probe used in this experiment contained both α and β sequences. Fig. 5 demonstrates that the human fibroblasts in the control experiment contained no detectable human globin mRNA sequences in the cytoplasmic RNA. In contrast,
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Humphries, Windass & Williamson (1976) using much larger RNA input levels than tested in our experiments, found a small amount of hybridizable globin mRNA in nuclear and cytoplasmic RNA derived from non-erythroid cells, both from animals and from tissue culture lines. We did not analyse nuclear RNA in our experiments. Groudine, Holtzer, Scherrer & Therwath (1974) and Groudine & Weintraub (1975) found no globin mRNA in nuclear or total cellular chick non-erythroid tissues. A very low level of globin gene transcription in non-erythroid tissues may occur in some species and not in others.

The cross-hybridization between mouse and human mRNAs and cDNAs was very low. This low background allowed clear-cut interpretation of the heterokaryon data. These cells contained no human $\alpha$- or $\beta$- mRNA sequences, but did contain mouse globin mRNA.

In summary, we found that heterokaryons formed from mouse erythroleukaemia cells and human fibroblasts did not synthesize detectable human $\gamma$ or $\alpha$ globin chains. In addition, human $\beta$ or $\alpha$ globin mRNA sequences were not produced in the heterokaryons. The combination of these results lead us to the conclusion that human globin gene expression was not activated in these heterokaryons. The fused cells were competent, as demonstrated by the fact that they contained mouse globin mRNA and synthesized mouse globin chains. Our experiments did not distinguish between the utilization of preformed globin mRNA, which was present in the erythroblasts when they fused with the fibroblasts, and the production of new mouse mRNA after the heterokaryons had formed.

The experiments described in this report show that heterokaryons formed readily between mouse erythroleukaemia cells and human fibroblasts. Globin synthesis occurred in these heterokaryons for up to three days following fusion. Despite the use of erythroblasts from a tumour cell line, and with an excessive number of chromosomes relative to the diploid normal fibroblasts, there was no apparent induction of the globin genes in the fibroblast partner in these heterokaryons. Further experiments will be directed at evaluation of the effect of exogenous inducing agents on these heterokaryons. The role of cell division in gene induction will be explored by similar investigation of hybrid cells.

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