LOCALISATION OF CELLULAR GLOBIN MESSENGER RNA BY *IN SITU* HYBRIDISATION TO COMPLEMENTARY DNA

P.R. HARRISON, D. CONKIE and J. PAUL
The Beatson institute for Cancer Research, 132 Hill Street, Glasgow G3 6UD, Scotland

and

K. JONES
Department of Epigenetics, University of Edinburgh, West Mains Road, Edinburgh EH9 3JN, Scotland

Received 22 March 1973

1. Introduction

An important aspect of the control of cellular differentiation is the sequential activation of genes, leading to synthesis of specific messenger RNA's (mRNA's) specifying proteins characteristic of a particular differentiated cell. The problems involved in elucidating these complex biological changes are basically two-fold: i) to detect in a given cell type minute amounts of specific mRNA's and ii) to correlate changes in amounts of specific mRNA's with changes in the differentiated state.

This report describes a general method which allows detection of minute traces of a specific mRNA in cytological specimens of cells. The method is applied to detect globin mRNA in erythroid cells. We have shown [1, 2] that a reasonably faithful DNA copy (cDNA) of globin mRNA can be obtained by transcribing mouse reticulocyte 9 S RNA by reverse transcriptase. Using this cDNA as a probe, the conventional *in situ* hybridisation technique is extended to detect globin mRNA's in the developing foetal liver and in Friend-virus transformed cells treated with dimethyl sulphoxide (DMSO).

2. Methods

Foetal liver cells were obtained by routine methods [3]. Clone 707 Friend cells (originally supplied by Dr. Charlotte Friend, Mount Sinai School of Medicine, New York), were grown in Ham's F12 medium supplemented with MEM amino acids and 16% horse serum.

cDNA was obtained essentially as described previously [2] except that unlabelled dATP and dGTP (at a concentration of 500 μM) replaced labelled dATP and dGTP, and reverse transcriptase of activity 100 units/ml was used. The purified cDNA had a specific activity of about 26×10^5 dpm/μg and mean size 120,000 daltons.

Cytocentrifuge preparations of cells, fixed in ethanol/acetic acid (3:1), were prepared for *in situ* hybridisation by treatment with 0.2 N HCl for 20 min at 20°, followed by dehydration through 50%, 70%, 90% and absolute ethanol. cDNA was dissolved (about 4×10^7 dpm/ml) in either (a) 0.3 M NaCl, 0.03 M citrate (2 × SSC) or (b) 3 × SSC containing 40% formamide (redistilled or Fluka). About 3 μl of cDNA were applied to each slide, covered and sealed before incubating (a) at 60° for 6–18 hr or (b) 44° for 18 hr. The preparations were uncovered, washed in 2 × SSC at 20°, treated for 1–2 hr at 55° in 2 × SSC, then dehydrated. In some cases, the preparations were incubated with S1 nuclease in place of the

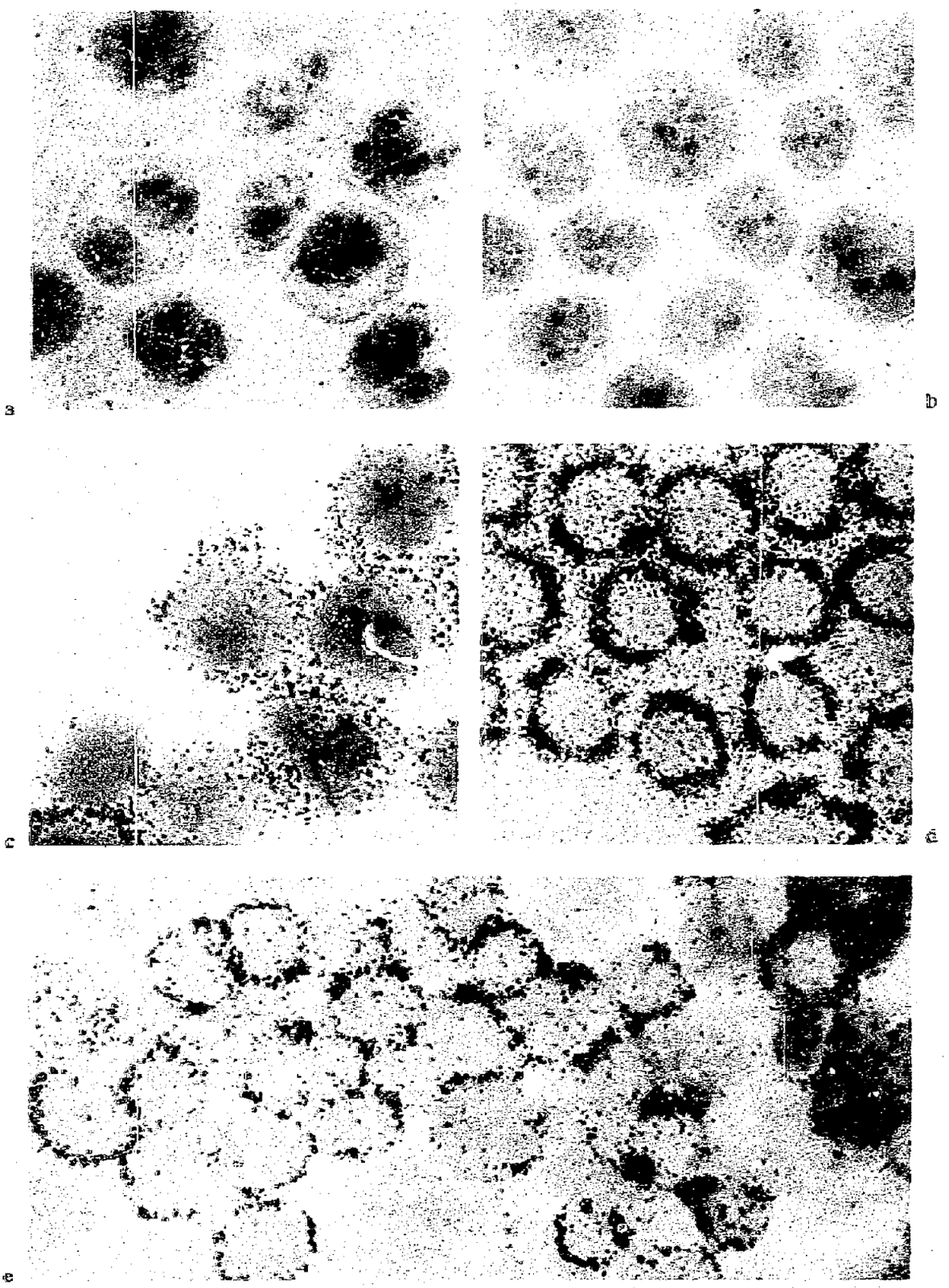

Plate 1. a = LS cells; b = Friend 707 cells. c, d = Friend 707 cells after growth for 3 days in 1.5% DMSO. e = 13.5 day foetal liver cells. All autoradiographs were exposed for 10 weeks.

wash in 2 X SSC at 55°. Autoradiographs were prepared and exposed for 3–26 weeks as necessary.

3. Results

The results of *in situ* hybridisation of globin cDNA to various cells is illustrated in plate 1. Only background labelling is observed over LS cell preparations even with exposure of 6 months. However, most cells derived from 13.5-day foetal liver are heavily labelled, mainly in the cytoplasm. Labelled cells comprise both immature (mainly basophilic erythroblasts, but also some proerythroblasts, as judged by size) and mature cells (polychromatic and orthochromatic erythroblasts). However, labelling of reticulocytes is rarely observed, either in these preparations of foetal liver or in cytocentrifuge preparations of reticulocytes from phenylhydrazine-treated mice. This anomaly is probably due to the fragility of reticulocytes under the conditions of *in situ* hybridisation. Heavier labelling is obtained by hybridising cDNA to foetal liver cell preparations at 44° in 3 X SSC/formamide rather than in 2 X SSC at 60°. The morphology and staining of foetal liver preparations after the latter procedure is also impaired. However, under both conditions, no loss of labelled RNA from either nucleus or cytoplasm has been detected autoradiographically in control cells labelled *in vitro* with [^{3}H]uridine.

Friend cells (clone 707) show a very low labelling after *in situ* hybridisation to globin cDNA. However, after growth of these cells in 1.5% DMSO for 3 days, very heavy labelling of almost all the cells is observed. As with foetal liver cells, this labelling is predominantly cytoplasmic. This increase in *in situ* hybridisation of globin cDNA occurs before the increase in haemoglobin content which reaches a maximum after treatment of these cells with DMSO for about 5 days (D.C., unpublished results). However, after longer exposures (about 6 months), untreated Clone 707 Friend cells show a distinct labelling, in contrast to LS cells treated identically. Control experiments show that there is little loss (perhaps 20%) of [^{3}H]uridine-labelled RNA from either nucleus or cytoplasm after *in situ* hybridisation in SSC/formamide at 44°. However, in contrast to foetal liver cells, a considerable loss (about 50%) of cytoplasmic label, but not nuclear label, occurs after hybridisation in 2 X SSC at 60°. This loss may be due to actual loss of total cytoplasm or specific loss of RNA during the *in situ* procedures. Visible loss of cytoplasm is noticeable in certain cases, e.g. Friend cells treated with DMSO for 5–6 days.

The results described above are typical of several slides and experiments. In widely scattered fields on some slides, there was considerable variation in degree of labelling (c.f. plate 1 c & d). However, the same striking differences in labelling between the various cell types have been obtained independently in our two laboratories.

4. Discussion

Globin cDNA is known to be a reasonably faithful copy of globin mRNA ([1, 2] and P.R.H., unpublished data). Therefore, provided the observed *in situ* hybridisation of globin cDNA represents specific RNA–DNA hybridisation, then these experiments show that globin mRNA can be detected in fixed slide preparations of cells.

4.1. *Stringency of hybridisation conditions*

Globin cDNA was incubated (usually in 3 X SSC/40% formamide) with the denatured preparations at a temperature about 15° below the melting temperature of 40% G+C RNA–DNA hybrids [4]. Under these conditions, specific cDNA-globin mRNA hybrids are formed in true solution ([2], P.R.H., unpublished results). Furthermore, after hybridisation, the cell preparations were routinely stressed thermally at 55° in 2 X SSC or treated with a single-strand specific nuclease. Both these treatments should eliminate non-specific binding of cDNA.

4.2. *Specificity of hybridisation with respect to cell type*

In situ hybridisation of cDNA is only observed to normal erythroid cells (foetal liver) or haemoglobinised cells (Friend cells after treatment with DMSO) but not to non-erythroid cells (LS cells). An anomaly is the lack of *in situ* hybridisation of cDNA to reticulocytes, from which 9 S RNA (used to prepare globin cDNA) is isolated. This indicates that care must be taken in interpreting negative results obtained by this

technique. Certain cells may lose globin mRNA during fixation or *in situ* hybridisation. Alternatively, globin mRNA may be masked by protein, for example, high cellular concentrations of haemoglobin.

4.3. *Relevance of results*

These results illustrate primarily the validity of the *in situ* technique for locating at least one specific mRNA in cytological preparations of cells. Calculations based on the minimum grain count considered significant, the specific activity of cDNA and an assumed autoradiographic efficiency of about 10%, show that the method detects about 5×10^{-5} pg of globin mRNA in a given cell. It is impossible to measure directly what proportion of globin mRNA in a fixed cell hybridises to cDNA under these conditions *in situ*. A typical grain count of about 150 over three-day induced Friend cells exposed for seven weeks corresponds to about 8×10^{-4} pg of globin mRNA, which is about 0.008% of the total cellular RNA. This proportion is about one half of that estimated by hybridisation of globin cDNA to three-day induced Friend cell polysomal RNA in true solution [Gilmour, Windass and Paul, personal communication]. It is therefore clear that the technique is extremely sensitive. Coupled with the fact that measurements are made finally by microscopic observation on cytological preparations, this means that the technique is particularly valuable in locating minute traces of specific mRNA's in cells difficult to obtain either pure or in large quantities.

The biological implications of these and similar experiments in connection with erythroid cell development will be the subject of subsequent reports (in preparation). However, the present results show that in 13.5-day foetal liver, globin mRNA is detectable in some cells classed as proerythroblasts and in most basophilic erythroblasts. This indicates that, at this stage of foetal liver development, the globin genes probably begin to be activated in the earliest recognisable erythroid precursor cell. In terms of whether the globin genes are activated, the uninduced clone 707 cell appears to be equivalent to the proerythroblast of the normal erythroid cell series.

Acknowledgements

This work has been supported by grants from the Medical Research Council, the Cancer Research Campaign and the Muscular Dystrophy Group of Great Britain.

References

[1] P.R. Harrison, A. Hell and J. Paul, FEBS Letters 24 (1972) 73.
[2] P.R. Harrison, A. Hell, G.D. Birnie and J. Paul, Nature 239 (1972) 219.
[3] R.J. Cole and J. Paul, J. Embryol. Exp. Morph. 15 (1966) 245.
[4] J.O. Bishop, Biochem. J. 126 (1972) 171.