TRANSCRIPTION OF GLOBIN GENES IN RETICULOCYTE CHROMATIN

Daphne MARYANKA, Graham J. COWLING, James ALLAN, Stephen J. FEY, Piroska HUVOS and Hannah J. GOULD

Department of Biophysics, King's College, 26–29 Drury Lane, London WC2B 5RL, England

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1. Introduction

The use of exogenous RNA polymerase in studies of the transcriptional specificity of chromatin is based on two unproven assumptions:

(1) That the active genes in chromatin are in a unique conformation preferentially transcribed by the polymerase;

(2) That this conformation survives the process of isolation of the chromatin.

The further extension to the study of the fidelity of chromatin reconstitution rests on the additional precept that the specificity of transcription of reconstituted chromatin can be measured in relation to that of 'native' chromatin. The data presented here undermine at least one of these assumptions. We show that there is indeed little evidence for globin gene specificity in transcription of reconstituted reticulocyte chromatin.

2. Materials and methods

The procedures for chromatin reconstitution and transcription with mercurated UTP and *Escherichia coli* RNA polymerase and the purification, affinity chromatography and hybridisation analysis of the RNA products have been detailed in [1].

Nuclei from chicken reticulocytes were isolated as in [2], using Nonidet NP40 as the detergent, and stored at −70°C. Chromatin was freshly prepared by washing thawed nuclei twice with 10 mM Tris–HCl, 25 mM EDTA, (pH 7.5) and hand-homogenising the resulting pellet in cold distilled water. The swollen chromatin was then concentrated by centrifugation at 16 000 × *g* for 10 min. Endogenous nuclear RNA was isolated from stored nuclei by repeated phenol extraction and separated from DNA by CsCl density gradient centrifugation [3]. ^{125}I-Labelling of this RNA (spec. act. 10^6 cpm/μg) was by the method in [4].

Three independent methods were used to correct the amounts of globin RNA detected by affinity chromatography on thiopropyl–Sepharose [1] for cross-contamination by endogenous globin RNA.

Method A: A 'spillover' correction was obtained by adding a trace amount of the ^{125}I-labelled nuclear RNA to the transcription system either just before or just after transcription. The distribution of radioactivity after the usual RNA extraction and chromatography was determined, and the percentage of ^{125}I-label bound to the column was used to estimate the contamination of the bound RNA with endogenous globin RNA. This percentage of the total endogenous globin RNA was subtracted from the total bound globin RNA.

Method B: The ability of the mercurated globin RNA hybridised with complementary DNA to cause retention of the labelled cDNA on the affinity column was used to measure the fraction synthesised in vitro [5]. The RNA bound as usual to the column was precipitated with ethanol, dried and redissolved in 25 μl hybridisation buffer containing excess (1.8 ng) complementary DNA. After 24 h hybridisation in sealed capillaries, the samples were expelled into 0.5 ml S_1 nuclease buffer [1] and digested with 0.12 IU of the enzyme. The digestion was terminated by addition of 100 μl 10% lithium dodecylsulfate. An aliquot (150 μl) was immediately precipitated with trichloroacetic acid to determine the total radioactivity, while

the remainder was applied to a column of thiopropyl–Sepharose overlaid with a 1 mM layer of Chelex 100 resin (Biorad) and the cDNA radioactivity was monitored after elution in the usual way.

Method C: Actinomycin D was used to inhibit DNA-dependent RNA synthesis, and the remainder was taken as RNA-dependent synthesis [5]. This value was subtracted from the total obtained in the absence of actinomyin D for calculation of the fraction of globin RNA synthesised.

3. Results

The specificity of the endogenous chicken reticulocyte RNA polymerase is shown by the results of incubations of nuclei, as well as by the occasional chromatin preparations which displayed activity in the absence of added RNA polymerase (table 1). In both cases, total RNA synthesis was $\leqslant 1-2$ μg, and globin RNA synthesis only 0.2–0.6 ng, which represents on average barely a single round of transcription of the 3 adult chicken globin genes (one β, two α) known to be expressed in vivo. The specificity for globin RNA synthesis for the endogenous polymerase was thus some 500 ppm.

On addition of *E. coli* RNA polymerase, RNA synthesis is stimulated to widely varying degrees. Some typical examples of results from numerous experiments are shown in table 1: here 3–30 μg RNA product was obtained with two different chromatin preparations incubated in identical conditions. For the lower limit of total synthesis, the specificity was 10 times higher. In this case, the level of globin specificity approached at 100 ppm the level found for the endogenous enzyme at 500 ppm (table 1). The endogenous enzyme (polymerase II) was inhibited in this experiment by α-amanitin, so that the globin RNA was a product of the bacterial and not the endogenous enzyme. The trend throughout is of an inverse relation between total RNA synthesis and globin specificity.

To establish that the globin RNA detected in chromatin transcripts was synthesised in vitro, the following criteria have been suggested [5];

(i) Endogenous RNA should not copurify with the mercurated transcripts on the affinity column used to isolate the mercurated RNA.

(ii) The globin RNA hybridised with labelled complementary DNA should lead to retention of the cDNA on the affinity column.

(iii) Actinomyin D should inhibit the appearance of the globin RNA.

Table 2 shows how an analysis of the transcripts of 'native' and reconstituted chromatin stand in these

Table 1
Specificity of globin gene transcription in vitro

Template	Polymerase	Inhibitor	Total RNA (μg)	Bound RNA (μg)	Globin RNA				
					Unbound RNA		Bound RNA		
					cpm	ng	cpm	ng	ppm
Nuclei	Endogenous	–	2.0	1.25	1367–368	365	1714–264	0.65	520
			1.6	0.96	1299–368	201	906–228	0.62	630
Chromatin	Endogenous	–	1.0	0.31	421–228	40	887–264	0.28	900
			1.4	0.77	368–228	30	775–264	0.23	300
Chromatin	*E. coli*	–	17.7	6.6	384–228	33	1005–264	0.33	50
			33.4	16.8	488–228	53	634–264	0.17	10
Chromatin	*E. coli*	–	8.9	7.2	1524–227	26	1536–227	0.37	51
			7.7	6.7	1438–227	25	1455–227	0.32	48
Chromatin	*E. coli*	Rifampicin	0.26	0.19	2109–227	38	2451–227	0.44	5263
			0.11	0.06	1544–227	27	1539–227	0.27	4500
Chromatin	*E. coli*	–	3.2	1.5	1104–194	19	784–194	0.33	220
Chromatin	*E. coli*	α-Amanitin	3.2	1.6	1177–194	20	485–194	0.20	125

Table 2
Corrected estimates for the specificity of globin gene transcription

Chromatin template	%Hg UTP	Additions	Total RNA synthesis (μg)	Endogenous globin RNA (ng)	Retention by thiopropyl–sepharose				Globin RNA synthesis (ppm)			
					Total RNA (μg)	Globin RNA (ng)	^{125}I-Labelled RNA (%)	Hybrid cDNA (%)	Apparent	Corrected[a]		
										A	B	C
Native	100	–	3.9	14.2	2.9	0.37	–	24	125	103	30	–
Native	25	–	2.9	13.1	2.7	0.12	–	7	44	20	3	–
Reconstituted	100	–	79	20.0	68	0.25	–	21	3.7	2.2	0.8	2.9
Reconstituted	25	–	79	12.4	47	0.39	–	15	8.4	7.0	1.3	7.1
Reconstituted	25	Actinomycin (100 μg/ml)	4.3	n.d.	1.2	0.056	–	11	–	0	5.1	0
Reconstituted	25	^{125}I-Labelled RNA before transcription	33	4.2	21	0.036	0.22	–	1.7	0.7	–	–
Reconstituted	25	^{125}I-Labelled RNA after transcription	38	3.4	23	0.048	0.77	–	2.0	1.3	–	–

[a] See section 2

terms. The 0.5% 'spillover' correction obtained by following the distribution of ^{125}I-labelled RNA on affinity chromatography (method A) reduces the estimated globin RNA synthesis somewhat, but does not affect the conclusion that globin RNA was transcribed from the chromatin template in vitro. The tests of the RNA–cDNA hybrids (method B) lead to a larger correction, since $\leqslant$ 24% of the hybrid was bound by the affinity column. This value was reduced in proportion to the fraction of HgUTP in its mixture with UTP used for transcription. The corrected value for globin RNA synthesis from the chromatin template (30 ppm) which represents a lower limit is nonetheless significant. The use of actinomycin D (method C) leads to only minor corrections.

That endogenous globin RNA does not vitiate the estimations of mercurated globin RNA is also shown by the fact that the globin RNA detected by affinity chromatography bears no relation to the variable amount of this RNA present in chromatin over the range of 0–50 ng (fig.1).

To test whether *E. coli* RNA polymerase initiates the synthesis of globin chains on reticulocyte chromatin, we added rifampicin to block initiation. In the presence of rifampicin, total RNA synthesis was inhibited by $>$ 80%, but globin RNA synthesis not at all (table 1). We conclude that the globin RNA chains synthesised in vitro were not initiated by the bacterial enzyme. It is possible that RNA, initiated by the endogenous enzyme in vivo, was elongated by *E. coli* RNA polymerase in vitro.

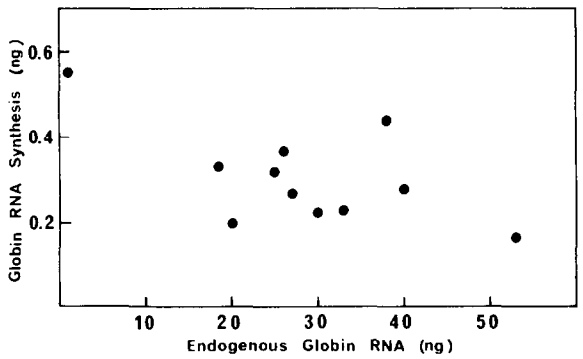

Fig.1. The apparent synthesis of globin RNA by *E. coli* RNA polymerase plotted against the endogenous globin RNA content of the chromatin template.

4. Discussion

There have been conflicting conclusions about the specificity of transcription of reticulocyte chromatin by *E. coli* RNA polymerase. According to one view [5–7] the transcription is essentially random, whereas other workers believe it to be globin specific [8–13]. A close examination of the published results suggests however that the disagreement is illusory. Thus the 10–20 ppm globin RNA in [6,7] was dismissed as insignificant in comparison to the values of 1000–2000 ppm obtained with reticulocyte nuclei. The others [8–11] however, judged rather similar values of 10–40 ppm globin RNA to be significantly higher than the level of random DNA transcription. The latter is ~0.5 ppm globin RNA, but we find that in practice DNA transcripts contain 1–4 ppm globin RNA. It may be noted that values varying over a 10-fold range, from 40 ppm [10] to 300 [11] ppm globin specificity, have been reported from the same laboratory. This accords with our findings (table 1). If the maximum globin gene specificity of chromatin is taken as 100 ppm, it is at most 2 orders of magnitude greater than that of DNA, but around an order of magnitude less than that of nuclei, usually reported as 1000–2000 ppm [14,15] (but see table 1).

Our experiments seem to show that the transcription of globin genes by the bacterial polymerase is real, and not due to any of the known potential artefacts. This also emerges from the results [13] using a new approach which probably obviates all the problems of endogenous RNA contamination. In all the other work cited here, globin RNA was estimated by hybridising unlabelled RNA with labelled complementary DNA. Instead unlabelled, cloned globin cDNA was used [13] to estimate labelled RNA; thus RNA synthesised in vitro was detected at the level of 100 ppm. However, our experiments with rifampicin suggest that the globin chains are not initiated by the bacterial enzyme. If this is so then the selectivity operates against globin gene transcription, and our findings oppose the accepted wisdom.

Our explanation of the apparent specificity in terms of elongation of preinitiated RNA chains is consistent with a number of observations. Thus:

1. Rifampicin removes the high background of random

RNA chain initiation and synthesis, allowing the considerable specificity of the elongation process to reveal itself (table 1).

2. The inverse relation between total RNA synthesis and globin specificity (noted also [11]) may reflect variable random initiation and synthesis superimposed on the base level of elongation. The level of initiation is probably a function of the extent of damage that the chromatin undergoes in the course of perparation and storage [16].
3. The number of globin gene transcripts is limited in our experiments to an average of ~1/gene, and the RNA product is small (~80 nucleotides [17]): further transcription and re-initiation may be blocked by inactive transcription complexes.
4. The low affinity of the labelled transcripts for the thiol-column compared with DNA transcripts (results not shown) may mean that only part of the RNA chain is mercurated. In most experiments some 50% of the UTP was mercurated, and the unmercurated nucleotide is preferentially incorporated. The uridine content of chicken globin message is ~17% and thus ~8.5% of the 80 nucleotide-long chains at most would be mercurated. If 1% mercuration is sufficient to bind the RNA to the column [18], the average length of the extensions catalysed by the polymerase would be $\geqslant 10$ residues.
5. Only ~25% of cDNA hybridised with 100% mercurated transcripts is retained by the affinity column, which may indicate either the proportion of unmercurated chains or the presence of relatively short extensions which result in marginal binding.
6. The ability of *E. coli* RNA polymerase to elongate nascent chains in chromatin has been documented in another system [19].

The conclusion here must not be over-generalised. The ovalbumin gene in chromatin from stimulated oviduct was calculated to be transcribed ~100-times in vitro [20]. However, other workers have been less fortunate with the same system [21]. Oviduct chromatin may be more active than reticulocyte chromatin, since RNA synthesis in reticulocytes is a small fraction of that in the earlier erythroid cell precursors [22]. The 'reticulocyte' systems used by various workers may also differ in activity. These have included, besides chicken reticulocytes [5,6,14], mouse foetal liver [8–11], tissue-cultured erythroleukemia cells [12,15] and rabbit bone marrow [13].

If our interpretation is valid for the chicken reticulocyte system, then the *E. coli* polymerase merely reveals the specificity of the endogenous enzyme, reflected in the steady-state distribution of its product in chromatin, and not any special conformation of transcriptionally active regions of chromatin per se. Whatever the reasons, it is clear that the globin RNA chains are not initiated by exogenous polymerase. Since such initiation may be blocked by inactive endogenous transcription complexes, we cannot tell whether the enzyme might otherwise recognise (and selectively transcribe) the 'active' template conformation. The value of *E. coli* polymerase as a probe for this conformation is thus rendered questionable. One cannot altogether rule out that the 'active' conformation is not preserved in isolated chromatin.

The above considerations would seem to invalidate reliance on transcriptional specificity of the 'native' material as a standard of reference for the fidelity of chromatin reconstitution. Demonstrable globin gene specificity on transcription of reconstituted chromatin relative to that on DNA would be of importance and would rehabilitate the bacterial polymerase as a valid conformational probe. On the other hand, the negative results obtained with reconstituted chromatin (table 2), can tell us little about the true functional specificity of this material. The conclusion of globin gene specificity for reconstituted mouse foetal liver chromatin [10], is not substantiated by the mere 15 ppm of globin RNA present in the transcripts [10], a value with which does not differ significantly from our own (table 2). There is thus little evidence in favour of restoration of native template activity following reconstitution. However, as we have indicated, the negative results do not prove that the opposite is the case.

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References

[1] Gould, H. J., Maryanka, D., Fey, S. J., Cowling, G. J. and Allan, J. (1978) Methods Cell Biol. 19, 387–422.

[2] Weinmann, T. G., Brendler, T. G., Raskas, H. J. and Roeder, R. G. (1976) Cell 7, 557–566.
[3] Ross, J. (1976) J. Mol. Biol. 106, 403–420.
[4] Commerford, S. L. (1971) Biochemistry 10, 1993–2000.
[5] Zasloff, M. and Felsenfeld, G. (1977) Biochemistry 16, 5135–5145.
[6] Crouse, G. F., Fodor, E. J. B. and Doty, P. (1979) Nucleic Acids Res. 6, 371–383.
[7] Konkel, D. A. and Ingram, V. M. (1978) Nucleic Acids Res. 5, 1237–1252.
[8] Gilmour, R. S. (1978) Phil. Trans. Roy. Soc. Lond. B 283, 379–380.
[9] Gilmour, R. S., Pays, E. and Donaldson, D. (1978) in: Gene Function, Proc. 12th FEBS Meet. (Rosenthal et al. eds) pp. 49–60, Pergamon, Oxford.
[10] Gilmour, R. S. (1978) in: The Cell Nucleus (Busch, H. ed) vol. 4, pp. 329–367, Academic Press, New York.
[11] Pays, E., Donaldson, D. and Gilmour, R. S. (1979) Biochim. Biophys. Acta 562, 112–130.
[12] Reff, M. E. and Davidson, R. L. (1979) Nucleic Acids Res. 6, 275–287.
[13] Jacquet, M., Levy, S. B., Robert, B. and Gros, F. (1977) Gene 1, 373–383.
[14] Fodor, E. J. B. and Doty, P. (1977) Biochem. Biophys. Res. Commun. 77, 1478–1485.
[15] Orkin, S. H. and Swerdlow, P. S. (1977) Proc. Natl. Acad. Sci. USA 74, 2475–2479.
[16] De Pomerai, D. I., Chesterton, C. J. and Butterworth, P. H. W. (1974) Eur. J. Biochem. 46, 461–471.
[17] Maryanka, D. and Gould, H. (1973) Proc. Natl. Acad. Sci. USA 70, 1161–1165.
[18] Dale, R. M. K. and Ward, D. C. (1975) Biochemistry 14, 2458–2469.
[19] Shih, T. Y., Young, H. A., Parks, W. P. and Scolnick, E. M. (1977) Biochemistry 16, 1795–1801.
[20] Tsai, M. J., Tsai, S. Y., Chang, C. W. and O'Malley, B. W. (1978) Biochim. Biophys. Acta 521, 689–707.
[21] Giesecke, K., Sippel, A. E., Nguyen-Huu, M. C., Groner, B., Hynes, N. E., Wurtz, T. and Schutz, G. (1977) Nucleic Acids Res. 4, 3943–3958.
[22] Longacre, S. S. and Rutter, W. J. (1977) J. Biol. Chem. 252, 273–283.