A Chicken Red Cell Inhibitor of Transcription Associated with the Terminally Differentiated State

Maggie E. Walmsley, Robin S. Buckle, James Allan, and Roger K. Patient
Division of Biomolecular Sciences Biophysics Section, Kings College, London WC2B 5RL, England

Abstract. When a red cell nuclear extract (RCE) from adult chickens was injected into Xenopus oocytes along with the chicken β globin gene, transcript levels were dramatically reduced compared to injection of DNA alone. The inhibitory action of the RCE was not specific to the β globin gene since the Herpes thymidine kinase and Xenopus 5S RNA gene transcript levels were similarly reduced. Transcriptional repression was observed even after passage of the RCE through oocyte cytoplasm to the nucleus. The inhibitory activity binds to DNA cellulose, which suggests that the inhibitor either binds to DNA or associates with DNA-binding proteins. Nuclease digestion of the chromatin assembled on injected β globin DNA revealed that inhibition was not associated with local changes in chromatin structure. Extracts from 9-d chicken embryonic erythroid cells, in which the endogenous β globin gene is actively expressed, did not inhibit transcription. The inhibitory activity is, therefore, restricted to transcriptionally quiescent, adult erythrocytes. Since the inhibitory effects were seen with both polymerase II and III directed genes, we speculate that the activity may be part of the extreme transcriptional repression which occurs in the terminally differentiated erythrocyte.

The mature chicken erythrocyte is a terminally differentiated cell in which synthetic activity is severely repressed. Unlike mammalian erythrocytes, the nucleus is retained (Davies, 1961) and within the nucleus, massive condensation of the chromatin is accompanied by near complete inactivation of RNA and protein synthesis (Cameron and Prescott, 1963; Madgwick et al., 1972; McLean and Madgwick, 1973). Only a residual, polymerase II-directed RNA synthesis remains (Ruiz-Carillo et al., 1974) which is attributed to chain elongation followed by rapid intranuclear degradation (Zentgraf et al., 1975). It has been assumed that changes in chromatin composition and structure, and in the total levels of RNA polymerases, represent the molecular basis for the extreme repression of the genome in these terminally differentiated cells (Kernell et al., 1971). The precise molecular events involved in the repression of the mature erythrocyte genome, however, remain unresolved.

The effects of chicken red cell extracts (RCE) on chicken β globin chromatin structure and expression in vitro have been reported. Nuclear extracts from chicken red cells in which the β globin gene is or has been actively transcribed (9-d embryo erythroid cells or mature erythrocytes from adults) induced nuclease hypersensitivity in the promoter of the gene (Emerson et al., 1989). The transcriptional activity of a cloned β globin gene in these extracts closely followed the in vivo developmental pattern of expression of the gene. Regulation of expression was dependent on β globin promoter binding factors whose composition change during red cell development. A promoter binding repressor was identified, the levels of which increase in mature erythrocytes. The relevance of these observations was limited, however, by the fact that the β globin 3' enhancer was nonfunctional in this in vitro system and the β globin gene was not assembled into chromatin.

The Xenopus oocyte offers an unusual opportunity to study transcription of microinjected genes complemented with protein extracts in an environment where the gene is assembled into chromatin. The use of Xenopus oocytes as an expression system is long established (Gurdon and Melton, 1981) and the process of chromatin assembly on DNA templates injected into intact Xenopus oocytes has been well characterized (Gargiulo and Worcel, 1983; Ryoji and Worcel, 1984; Ruberti and Worcel, 1986).

To determine the effects of chicken red cell nuclear extracts on the transcription of chicken β globin gene chromatin, we coinjected the gene and the extracts into Xenopus oocytes. We found that the chicken β globin gene, when injected alone into oocytes, was transcribed very efficiently and that coinjection of adult erythroid nuclear extracts led to a dramatic reduction in transcript levels. Extracts from 9-d embryonic red cells, however, did not repress transcript levels. Furthermore, the inhibitory activity in the adult extracts was not re-
stricted to the β globin gene but similarly affected another polymerase II-directed gene, the herpes thymidine kinase (TK) gene, and a polymerase III-directed gene, the Xenopus 5S RNA gene. The inhibitory activity may, therefore, be involved in the generalized transcriptional repression which occurs in the terminally differentiated chicken erythrocyte.

**Materials and Methods**

**Preparation of Nuclei from Chicken Red Cells**

**Adult Chickens.** Blood from a White Leghorn chicken was collected through gauze into an ice-cold solution of PBS (0.85% NaCl (w/vol) in 0.15 M phosphate buffer (pH 7.2) containing 1 mM EGTA, 0.2 mM PMSEF, and 25 U/ml heparin (Evans Medical, Langhurst West Sussex, England). All subsequent steps were performed at 4°C. Blood cells were sedimented at 2,000 g for 5 min in a centrifuge (model RC-5; Sorvall Instruments, Newton, CT) and washed three times in PBS containing 1 mM EGTA and 0.2 mM PMSEF. After aspiration of the white cell layer, the red cells were resuspended in a small volume of buffer I (10 mM Tris·HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 5 mM sodium butyrate, 1 mM DTT, 0.1 mM PMSEF) and then added dropwise to seven volumes of buffer II (buffer I plus 1% Triton X-100) and stirred for 15 min. Nuclei were sedimented at 2,000 g for 5 min and then washed twice in buffer I.

**9-10-day Embryos.** Blood was recovered from 100 9-10-day chick embryos and filtered through gauze to remove yolk and debris. Nuclei were prepared in the same manner as for adult erythrocytes except that cell lysis was effected by the addition of buffer I plus 0.2% Triton X-100 with mixing, with no incubation before nuclei sedimentation.

**Preparation of 0.15 M NaCl Nuclear Extracts**

Nuclei were prepared as described above from either adult or 9-10-day chicken erythrocytes and resuspended in a small volume of buffer I. 5 M NaCl was added dropwise to a final concentration of 0.15 M, and the nuclei were incubated at 37°C for 10 min with intermittent agitation. Nuclei were then pelleted by centrifugation at 10,000 rpm for 5 min in a centrifuge (model RC-5; Sorvall Instruments) at 4°C. The supernatant was carefully removed, aliquotted, and frozen at −70°C. Protein concentration was estimated by averaging the values determined by measuring the OD₂₈₀ and by the McKnight method (McKnight, 1977). In some cases, additional protease inhibitors (benzamidine, pepstatin A, aprotinin, and leupeptin) were included in the procedures for nuclear isolation, nuclear extract preparation, and DNA cellulose fractionation. We found no differences between the activity of such extracts compared to extracts prepared without the additional inhibitors in the assays used here.

**DNA Cellulose Fractionation of RCE**

RCE was diluted 1:3 with 10 mM Tris·HCl, pH 7.5, 1 mM DTT, 0.1 mM PMSEF to bring the salt concentration to 50 mM NaCl then loaded on a DNA cellulose column equilibrated in TND (10 mM Tris·HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 0.1 mM PMSEF). The column was washed sequentially with TND (flow-through fraction), TND+150 mM (NH₄)₂SO₄ (no fractions were collected), TND+250 mM (NH₄)₂SO₄ (250 mM fraction), and finally with TND+500 mM (NH₄)₂SO₄ (500 mM fraction). Samples corresponding to peak OD₂₈₀ values for each fraction were pooled and concentrated using Centricon microconcentrators (Amicon Corp., Danvers, MA). Each fraction was adjusted to 150 mM salt before microinjection.

**Oocyte Injection**

Supercil iodinated DNA for injection was preincubated with binding buffer (10 mM Tris·HCl, pH 7.5, 0.15 M NaCl, 3 mM MgCl₂, 1 mM DTT) or with RCE in binding buffer for 30 min on ice. 50 nl DNA or DNA-protein mixture was then injected into the oocyte nucleus. Isolation and injection of stage VI oocytes (Dumont, 1972) was as described (Waltmsley and Patient, 1987). Briefly, oocytes were treated with collagenase to remove ovarian follicular material and centrifuged to bring the nucleus to the cell surface. After injection, oocytes were incubated for 24 h at 19°C in modified Barth's saline (Gurdon and Wickens, 1983). Half of the healthy oocytes were selected for chromatin analysis as described below and the other half were snap frozen in liquid nitrogen and stored at −70°C. Total nucleic acid, for RNA analysis and monitoring supercil iodinated DNA template as a measure of injection success, was extracted from the snap frozen oocytes as described (Waltmsley and Patient, 1987).

**Chromatin Analysis**

12-15 pooled oocytes were prepared for DNAse I hypersensitivity analysis as described (Gargiulo and Worcel, 1983). Oocytes were gently homogenized in 500 µl KPS buffer (85 mM KCl, 5 mM Pipes, pH 70, 5.5% sucrose) and the homogenate removed avoiding any large debris. The homogenate was made up to 300 µl in MgCl₂ and CaCl₂, respectively, and then DNase I was added to 80 µl aliquots of homogenate over a concentration range of 0-15 µg/ml. Incubation was for 5 min at room temperature. The reaction was stopped by addition of EDTA to a final concentration of 10 mM. After digestion with 50 µg/ml RNase for 30 min at 37°C, SDS was added to 0.5% and the samples were digested with 200 µg/ml proteinase K at 35°C overnight. Samples were extracted with phenol and chloroform and then precipitated with ethanol. For the MspI digestion, samples obtained after oocyte homogenization were digested with 0.5 U of MspI for 5 min at 37°C. DNA was then purified as above. Samples were restricted with HindIII overnight, extracted with phenol and chloroform and loaded onto a 1% agarose gel containing ethidium bromide. Samples were transferred to nitrocellulose and hybridized with a 32P-labeled HindIII-MspI probe (Fig. 1 A) prepared by the mixed oligonucleotide priming technique (Feinberg and Vogelstein, 1984).

**SI Mapping Analysis**

**Chicken β Globin Transcription.** Total RNA from 1-3 oocytes and ∼1.3 ng (2 × 10⁵ cpm.) of single stranded Neol–Apal probe, 5' end labeled at the Neol site (Fig. 1 A), were precipitated with ethanol and the pellet dissolved in 25 µl formamide buffer (80% formamide, 0.4 M NaCl, 40 mM Pipes, pH 6.4, 1 mM EDTA). After denaturation at 95°C for 3 min, samples were quickly transferred to 55°C and incubated overnight. 300 µl cold SI buffer (50 mM sodium acetate, pH 4.5, 1 mM ZnSO₄, 0.1 M NaCl, 10 µg/ml denatured calf thymus DNA) containing 7,500 U nuclease SI (Boehringer Mannheim, U.K.) was added and the samples incubated for 2 h at 22°C. After ethanol precipitation, samples were analyzed by electrophoresis on a 6% denaturing polyacrylamide gel. Microheterogeneity of initiation at the cap site gives rise to a doublet both here and in the primer extension assay (see below).

**Thymidine Kinase Transcription.** The probe used was a single stranded 250-ntd PvuII–BglIII fragment, spanning the thymidine kinase (TK) cap site, 5' end labeled at the BglIII site. Methods were the same as those listed above except that hybridization was at 45°C.

**Primer Extension**

Primer extension analysis of steady-state RNA synthesized in injected oocytes was performed as described (Williams and Mason, 1985). The primer was an oligonucleotide, 18 nucleotides long, complementary to sequences from +55 to +72 in the first exon of the chicken β globin gene. 5' labeled primer (0.1 pmol of 5' ends) was hybridized to an aliquot of total RNA representing one to three injected oocytes in 0.4 M NaCl, 10 mM Pipes, pH 6.4 at 52°C for 2-2.5 h in a final volume of 10 µl. 90 µl of primer extension mix (55 mM Tris·HCl, pH 8.2, 11 mM DTT, 67 mM MgCl₂, 278 µg/ml actinomycin D, 0.55 mM dNTPs, 10 U reverse transcriptase) was added to each sample and primer extension was performed for 1 h at 42°C. After ethanol precipitation, samples were analyzed by electrophoresis on a 6% denaturing polyacrylamide gel.

**5S RNA Transcription**

Plasmid DNA pXial1, containing the Xenopus 5S RNA gene, was incubated on ice either with binding buffer or with RCE for 30 min and injected into oocytes along with α-32P-UTP. After an overnight incubation at 19°C, labeled RNA was extracted from injected oocytes and separated on a 6% denaturing polyacrylamide gel.

**Southern Injection Controls**

Samples representing total nuclear acid extracted from a single injected oocyte were run on a 1% agarose gel without ethidium bromide in a Tris-phosphate buffer (0.02 M Tris·HCl, pH 7.3, 0.036 M Na₂HPO₄, 0.1 mM
An Adult Chicken RCE Inhibits Chicken β Globin Transcript levels

When a chicken β globin gene construct, pCARB4.4 (Fig. 1 A), was injected alone into Xenopus oocytes, high levels of capsite initiated transcripts were detected by S1 mapping. These were accompanied by transcripts initiated upstream of the capsite, represented by protection of full-length probe (Fig. 1 B, lane 1). This pattern of transcription is typical of RNA polymerase II genes injected into Xenopus oocytes (Jones et al., 1983; Bendig and Williams, 1984; Partridge et al., 1984; Walmsley and Patient, 1987). Like the Xenopus β globin gene (Walmsley and Patient, 1987), transcription of the chicken β globin gene is highly efficient, being only two orders of magnitude below that calculated for in vivo expression (Weintraub, 1985). When a low salt extract from adult chicken erythrocyte nuclei was co-injected with the β globin gene after a period of preincubation, capsite initiated transcripts were eliminated and upstream initiations were greatly reduced (Fig. 1 B, lanes 2 and 3). This result was consistently obtained in several different experiments using a number of extract preparations. This effect was not the result of variation in injection success as shown by the levels of supercoiled DNA template recovered from the same pool of oocytes used for RNA analysis (Fig. 1 B, bottom). Supercoiled DNA is known to be the topological form which supports RNA polymerase II transcription in injected oocytes (Harland et al., 1983). Injected DNA which misses the nucleus and is deposited in the cytoplasm, becomes nicked, linearized, and finally degraded (Gurdon and Melton, 1981). Thus, recovery of supercoiled DNA from injected oocytes is an accurate measure of injection success. Fig. 1 B, bottom, demonstrates that the majority of templates rescued from oocytes after an overnight incubation were supercoiled (lanes 1–3) and confirms that the inhibitory effect of the adult RCE was not caused by a variation in injection success.

The Adult RCE Contains Neither Ribonuclease nor Deoxyribonucleolytic Activity

Two trivial ways in which the adult RCE could inhibit RNA accumulation are by template damage or by RNA degradation. We have already demonstrated that equivalent amounts of supercoiled templates were recovered from oocytes both in the presence and absence of RCE (Fig. 1 B, bottom). Thus any template damage incurred in the preincubation of DNA and RCE was repaired before recovery of the injected plasmids from the oocyte. However, damage to the template before injection could prevent assembly of an active transcription complex. To determine the extent of nucleolytic activity in the adult RCE, the integrity of the DNA was examined after incubation with extract. pCSGSI (Fig. 1 A) was incubated with extract on ice for 2–3 h, the normal length of an injection experiment. After extraction, the DNA was analyzed by agarose gel electrophoresis. When compared to the starting material, the ratio of supercoiled DNA to nicked circle and linear forms was essentially unaltered by incubation with RCE (Fig. 2 A, compare lanes 4 and 6). Thus the adult RCE contained very little deoxyribonuclease activity and template damage is unlikely to explain the inhibitory effect on RNA accumulation.

To test for RNA degradation, poly(A+) RNA from chicken reticulocytes was incubated with two individual adult RCE preparations at room temperature. Samples were removed after 1, 4, and 16 h and surviving RNA was analyzed by primer extension and compared to a sample incubated without extract (Fig. 2 B, compare lanes 4–9 with lanes 1–3). Clearly, the poly(A+) RNA survived extensive incubation with adult RCEs, showing that the extracts were very low in ribonuclease activity. This result suggests that the inhibitory activity of the adult RCE acts at the level of RNA production rather than message stability.

Inhibition of Transcript Levels by the Adult RCE Is not β Globin Specific

To determine the specificity of the inhibitory effect of the adult RCE on RNA production, we co-injected the β globin template with another gene transcribed by RNA polymerase II (the Herpes simplex virus TK gene) and also with a gene transcribed by RNA polymerase III (the Xenopus 5S ribosomal RNA gene). RNA from the same batch of oocytes shown in Fig. 1 B, which had been co-injected with the chicken β globin gene and the herpes TK gene, was probed for TK transcripts. TK transcription was inhibited to an extent equivalent to β globin (Fig. 3 A, lanes 1–3). Injection controls are as for Fig. 1 B, bottom. The RCE also inhibited the production of 5S RNA transcripts when the Xenopus 5S RNA gene was co-injected with the chicken β globin gene (Fig. 3 B, lanes 1 and 2). Thus the inhibitory effect of the adult RCE was not β globin specific.

Neither Chromatin Structural Changes Nor Binding of Nonspecific DNA-binding Proteins Mediate the Inhibitory Action of RCE

Salt extracts of the type used here contain a large number of specific and nonspecific DNA-binding proteins (Emerson et al., 1985; Plumb et al., 1985; Lewis et al., 1988; Jackson et al., 1989; Gallarda et al., 1989). The lack of gene specificity exhibited by the inhibitory activity raised the possibility that masking of the template by nonspecific DNA-binding proteins might be responsible. To determine if this was occurring, we monitored the chromatin structure of the injected templates. This analysis was of additional interest since similar extracts have been shown to confer DnaseI hypersensitivity to β globin gene chromatin reconstituted in vitro (Emerson and Felsenfeld, 1984).

Plasmid DNA injected into oocytes is assembled into minichromosomes, the DNA being rapidly relaxed by endogenous topoisomerases, and then supercoiled by wrapping into nucleosomes (Miller and Mertz, 1982; Ryoji and Worcel, 1984). Thus recovery of supercoiled templates after nuclear injection indicates successful chromatin assembly (Fig. 1 B, bottom).

In a number of experiments, the structure of the chromatin assembled on pCARB4.4 in injected oocytes, in the presence and absence of RCE, was analyzed using DnaseI or MspI as structural probes. The sites of enzyme cleavage were de-
Figure 1. Effects of adult RCE on chicken β globin transcript levels. (A) Diagram of pCARB4.4, indicating the HindIII–MspI DNA probe used for indirect end labeling, the ApaI–NcoI probe used for S1 mapping, and the SacI–NcoI fragment used for the construction of pCβGS1. pCARB4.4 consists of the chicken β globin-containing 4.4-kb EcoRI–BamHI fragment from pCAβG1 (McGhee et al., 1981) inserted into a 2.7-kb EcoRI–BamHI fragment from pBR322 from which the "poison" sequences have been deleted. This construct contains β globin sequences ~1 kb upstream of the capsite and 2 kb downstream of the poly (A*) site including the β globin 3' enhancer. pCβGS1 was constructed by inserting a 540-bp SacI–NcoI fragment from the β globin gene promoter into SacI–NcoI cleaved vector pE49 (Marsh, 1989). (B) Analysis of chicken β globin RNA produced in oocytes co-injected with chicken R globin gene (pCARB4.4) and the herpes thymidine kinase gene in the presence and absence of RCE. S1 mapping analysis (top). Lane M, molecular weight size marker, 3' end-labeled pAT153/HpaII digest yielding fragment sizes of 244 + 240 doublet, 219, 205, 192 (faint), 162 doublet, 149 (faint), 124, 112 (faint), 92, 78, and 69 ntds; lane P, 123-ntd NcoI–ApaI single stranded DNA probe 5' end labeled at the NcoI site, lanes 1-5, RNA from two oocytes injected with (lane 1) 3 ng pCARB4.4, (lane 2) 3 ng pCARB4.4 + 62.5 ng RCE, (lane 3) 3 ng pCARB4.4 + 125 ng RCE, (lane 4) RNA from two uninjected oocytes, (lane 5) 5 ng poly (A*) RNA from chicken reticulocytes; fP, full length probe. The position of β globin cap sites is indicated.

Southern injection controls (bottom). 1-3, DNA recovered from the same samples as 1-3 above; (M and M'), un.injected pCARB4.4 markers indicating the positions of supercoiled (sc), nicked circular (nc) and linear (lin) topological forms. The filter was probed with a fragment internal to the chicken β globin gene.
Figure 2. Adult RCE contains neither ribo- nor deoxyribonucleolytic activity. (A) Integrity of DNA templates after incubation with RCEs. DNA/protein (1:40) mixes were separated on a 1% agarose gel after incubation on ice for 2–3 h. Lanes 1–5, pCßGSI incubated with (lane 1) flow through fraction from DNA cellusose column, (lane 2) 250 mM salt eluate, (lane 3) 500 mM salt eluate, (lane 4) unfraccionated adult RCE, (lane 5) BSA, (lane 6) nonincubated pCßGSI starting material. Nicked circular (nc), supercoiled (sc), and linear (lin) forms of the DNA are indicated. (B) Test for ribonuclease activity in crude adult RCEs. 2 ng poly(A) RNA from chicken reticulocytes was incubated with 120 ng adult RCEs or binding buffer at room temperature. After 1, 4, and 16 h, one third of each sample was removed and 5 µg tRNA carrier was added. Samples were treated with phenol/chloroform, chloroform, ethanol precipitated, and then analyzed by primer extension using an oligonucleotide primer corresponding to nucleotides +55 to +72 relative to the capsite of the chicken β globin gene. Lanes 1–3, surviving poly(A) RNA after incubation with binding buffer for 1 (lane 1), 4 (lane 2), and 16 h (lane 3); lanes 4–6 as for lanes 1–3 except that poly(A) RNA was incubated with RCE1; lanes 7–9 as for lanes 1–3 except poly(A) RNA was incubated with a second extract preparation RCE2, lane M, 2 ng poly(A) RNA from chicken reticulocytes. The position of β globin capsites is indicated.

Inhibition of Transcript Levels by the RCE Is Mediated by a DNA–Cellulose-binding Activity which Migrates to the Nucleus

The lack of gene specificity for the inhibitory activity (Fig. 3), and the fact that inhibition was detected without significant changes in chromatin digestion patterns (Fig. 4), raised the possibility that binding to DNA is not involved. To address this question, the adult RCE was fractionated on DNA cellulose and the fractions were tested for inhibition of RNA accumulation by coinjection with a β globin template into the oocyte nucleus. As controls, the β globin gene was coinjected with unfraccionated RCE or with an equivalent amount of BSA.

The inhibitory activity present in the unfraccionated RCE (Fig. 5, lane 2 compared to lane 1) clearly bound to the column and was eluted with 250 mM salt (lane 5). Neither the flowthrough fraction nor the 500 mM salt elution contained very much inhibitory activity (lanes 4 and 6). The protein/DNA incubation mixes were monitored for possible template damage. All protein fractions were found to be low in deoxyribo–nucleolytic activity (Fig. 2 A, lanes 1–3, and lane 5). The retention by DNA cellulose suggests either that the RCE inhibitory activity functions as a DNA-binding protein or as DNA-binding proteins.

Nuclear proteins injected into oocyte cytoplasm have been shown to migrate into the nuclei within 1–2 h (Dingwall and Laskey, 1986; Leonard, 1989). We reasoned, therefore, that if the inhibitory activity functions as a DNA-binding protein or by associating with DNA-binding proteins, then we should be able to demonstrate the inhibition by injecting RCE into the cytoplasm. At the same time, we would effec-
Figure 3. The inhibitory effect of adult RCE is not \( \beta \) globin specific. (A) Effects of adult RCE on herpes TK gene transcripts. The samples used in Fig. 1 B were probed for herpes TK RNA accumulation by Sl mapping (Materials and Methods). Lane P, single-stranded 250 nt PsuII-BglII probe; lanes 1-3, RNA from three oocytes injected with 150 pg TK gene (lane 1), 150 pg TK gene + 62.5 ng RCE (lane 2), 150 pg TK gene + 125 ng RCE (lane 3); lane 4, RNA from three uninjected oocytes; lane M molecular weight size marker, 3' end-labeled pAT153/HpaII digest yielding fragment sizes 634, 494, 407, 244 + 240 doublet, 219, 205, 162 doublet, 124, 92, 78, and 69 ntds. fp is full-length probe. The position of the TK cap site is marked. Southern injection controls for this coinjection experiment are shown in Fig. 1 B, bottom. (B) Effect of adult RCE on Xenopus 5S ribosomal RNA levels. Injection and RNA analysis were as described in Materials.
tively fractionate the extract by selecting for nuclear proteins free of cytoplasmic contaminants. RCE was injected into oocyte cytoplasm followed 3.5 h later by injecting DNA into the nucleus. We found that the inhibition persisted even after passage of RCE through the cytoplasm (Fig. 5, compare lanes 1 and 3). Thus the inhibitory activity migrates from cytoplasm to nucleus.

**RCEs Prepared from 9-d Embryos do not Inhibit β Globin Transcript Levels**

Adult chicken erythrocytes are transcriptionally quiescent, with globin gene transcription occurring at earlier stages of erythroid differentiation (Landes et al., 1982). Thus, the inhibition of transcript levels by the adult RCE could be related to the terminally differentiated state. To test this possibility, nuclear extracts were prepared from 9-d embryonic red cells, which are transcriptionally active. When such an extract was co-injected into oocyte nuclei along with the β globin gene, no inhibition of β globin transcript levels was observed, whereas equivalent amount of an adult RCE did reduce RNA transcripts in the same experiment (Fig. 6, lanes 1–3). Since these cells contain large amounts of β globin mRNA, the 9-d extract was co-injected, as a control, with plasmid vector (pAT153) to ensure that the positive β globin RNA signal (Fig. 6, lane 3) did not arise from mRNA contaminating the RCE. No β globin RNA was detected in this sample (Fig. 6, lane 4) and the Southern control showed that the injection had been successful (Fig. 6, bottom, lane 4). Thus, an RCE prepared from 9-d embryos in the same way as that from adults did not inhibit RNA production. This experiment was repeated four times with the same result. It therefore appears that the inhibitory activity is restricted to transcriptionally quiescent, mature adult erythrocytes.

**Discussion**

We have detected an inhibitory activity in nuclear extracts from adult chicken erythrocytes which, when co-injected with the chicken β globin gene, brings about a reduction in RNA transcript levels. This effect is also observed for the herpes TK gene and the Xenopus 5S rRNA gene and is not, therefore, gene specific. The fact that the inhibitory activity persists after passage through the oocyte cytoplasm suggests a biological role within the nucleus.

We do not know precisely at which level of β globin expression the inhibitor has its effect. We have assumed that the effect is transcriptional rather than acting at the stages of processing or transport of RNA for the following reasons. In the experiment shown in Fig. 2, the β globin template used was pCβCS1 (Fig. 1 A), which only contains β globin sequences between nucleotides −460 and +80 relative to the cap site. The inhibitory effect, therefore, can be mediated by the promoter alone, indicating that splicing is not involved.

Furthermore, since our RNA analyses were performed on total cellular RNA, nuclear and cytoplasmic, inhibition of RNA transport from the nucleus would not have been detected. That we frequently observe complete elimination of the RNA signal in the presence of adult RCE, suggests that the inhibition acts at the level of generation of transcripts rather than affecting transport to the cytoplasm.

The inhibitory activity is retained on DNA cellulose (Fig. 5) but chromatin analysis shows that the RCE does not alter the DNasel digestion pattern of injected DNA templates (Fig. 4 A). Furthermore, although protection against Mspl cleavage was observed in the presence of RCE, its relief by nonspecific DNA competition did not lead to a lifting of the transcriptional repression (Fig. 4, B and C). Therefore, if transcriptional inhibition by the RCE does involve binding to DNA, it does not result in major disruption of the bulk of the chromatin templates. Repression by H1 or H5 linker histone is unlikely since these would not elute from DNA cellulose at the 250 mM salt required to recover the inhibitory activity. A protein, termed PAL, that binds to a specific DNA sequence has been identified in chicken erythroid cells as a repressor of β globin gene transcription in vitro (Emerson et al., 1989). This protein is unlikely to be responsible for the inhibition we observe since the RCE also suppresses expression of the TK and 5S genes, which contain no known PAL binding sites. The retention of the inhibitor on DNA cellulose may indicate that the activity associates with DNA-binding proteins, rather than binding DNA itself. Since the three genes that we have tested have no known DNA sequences, transcription factors or even RNA polymerase in common, the inhibitor would appear to have multiple targets, either on the DNA or on the proteins bound to it. Further fractionation of the RCE will provide more information on the nature of the inhibitor.

Whereas, in an in vitro system for chromatin assembly based on Xenopus oocyte extracts, nuclear extracts from chicken red cells were shown to induce hypersensitivity in the promoter of the chicken β globin gene (Emerson and Felsenfeld, 1984), we have found no such effect in the intact oocyte. In our chromatin analysis (Fig. 4), we found a lack of hypersensitivity in the β globin promoter even in the absence of the RCE when the gene is transcriptionally active. A number of studies have demonstrated that transcription in the oocyte is independent of the upstream sequences required for in vivo expression and can occur from templates driven only by TATA box (Mohn et al., 1986; Schorpp et al., 1988; Old et al., 1988). Thus, the transcription we observe in the oocyte in the absence of hypersensitivity may reflect its lack of dependence on the β globin upstream sequences. These considerations suggest that the inhibition acts via an effect on the basic transcriptional machinery.

Transcriptional shut down of the terminally differentiated erythocyte is accompanied by chromatin condensation.

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and Methods. RNA analysis (top). Lane M, molecular weight marker, 3' end-labeled pAT153/Hpal digest yielding sizes 162 doublet, 149, 92, 78, and 69 mts; lanes 1 and 2, RNA from a single oocyte injected with 1 ng pCARB4.4, 1 ng pXis11, 40 nCi a-32P-UTP and lane 1 binding buffer; lane 2, 40 ng adult RCE; lane 3, RNA from a single uninjected oocyte; lane 4, 5 ng poly(A)+ RNA from chicken reticulocytes. The positions of the 150 nt 5S RNA transcript and β globin capsites are marked.

Southern injection controls (bottom). Lanes M and M', un.injected pCARB4.4 markers showing the position of supercoiled (sc) and nicked circle (nc) DNA (M) and linear (lin) DNA (M'); Lanes 1 and 2, pCARB4.4 DNA rescued from samples 1 and 2, top.
Figure 4. Examination of the chromatin structure of β globin template injected in the presence and absence of adult RCE. (A) DNase I analysis. pCARB4.4 was preincubated either with buffer or with RCE at the protein/DNA ratios indicated, and injected into Xenopus oocytes. Minichromosome samples from 12-15 oocytes were digested with DNase I and processed as described in Materials and Methods. The right hand panel represents the DNase I digestion pattern of naked supercoiled pCARB4.4 DNA. The position corresponding to the hypersensitive site found in chicken erythrocytes (McGhee et al., 1981) is marked (bracket). M, EcoRI/Hind III digest of phage lambda DNA yielding fragment sizes 5.1 + 4.9 doublet, 4.3, 3.5, 2.0 + 1.9 doublet, 1.6, 1.3, 1.0, 0.8, and 0.6 kb. (B) MspI analysis. pCARB4.4 was injected into oocytes either alone, or in the presence of RCE (protein/DNA, 20:1), or in the presence of RCE (protein/DNA, 20:1) and a 35-fold molar excess of vector competitor DNA. Minichromosome samples obtained from batches of 12-15 oocytes were digested with MspI and processed as described in Materials and Methods. Lane 1, chromatin assembled in the absence of RCE; lane 2, chromatin assembled + RCE; lane 3, chromatin assembled + RCE + 35-fold molar excess of nonspecific competitor DNA; lane 4, EcoRI/Hind III digest of phage lambda DNA. Arrows indicate the hypersensitive MspI sites found in chicken erythrocyte nuclei (McGhee et al., 1981). (C) RNA analysis. β globin RNA synthesized in oocytes injected as described in B was assayed by primer extension using an oligonucleotide corresponding to nucleotides +126 to +146 relative to the β globin cap site. Lane C, 5 ng poly(A+) RNA from chicken reticulocytes; lanes 1-3, RNA from two oocytes injected with 1 ng pCARB4.4 and buffer (lane 1), 20 ng crude adult RCE (lane 2), and 20 ng crude adult RCE plus 20 ng of nonspecific vector competitor (lane 3). The position of the β globin cap sites is marked (bracket). Recovery of DNA in the chromatin analysis served as injection controls (B, lanes 1-3). The level of competitor was chosen to maximize competition for nonspecific DNA binding proteins in the RCE, whilst minimizing the effects on oocyte transcription. The small reduction in transcript levels in lane 3 compared to lane 2 reflects this. Significant competition of transcription in the highly active oocytes used in this experiment (lane 1) requires substantially higher levels of competitor DNA (Walmsley and Patient, 1987; and unpublished results).
Whereas it has been assumed that chromatin condensation per se is responsible for transcriptional repression, the results presented here suggest that other mechanisms may be involved. Our results suggest that, in the terminally differentiated erythrocyte, an inhibitory activity exists which acts directly on the transcriptional machinery to close down all RNA production.

Figure 5. Effects on β globin transcript levels of adult RCE introduced into oocytes via the nucleus or cytoplasm and after fractionation over DNA cellulose. pCβGSI was preincubated on ice for 30 min with RCE at a protein/DNA ratio of 40:1 then injected into the oocyte nucleus. Alternatively, crude RCE was injected into oocytecytoplasm followd 3.5 h later by injection of pCβGSI into the nucleus. Primer extension analysis (top). Lanes M, molecular weight marker, 3' end-labeled pAT153/HpaII digest showing fragment sizes 78 and 69 nt; lanes 1-6, RNA from a single oocyte injected with 0.5 ng pCβGSI and 20 ng BSA (lane 1), 20 ng unfractionated adult RCE via the nucleus (lane 2), or via cytoplasm (lane 3), 20 ng flowthrough fraction (lane 4), 20 ng 250 mM salt eluate (lane 5), 20 ng 500 mM salt eluate (lane 6); 5 ng poly(A) RNA from chicken reticulocytes (lane 7). Southern injection control (bottom). Lanes M and M', un.injected pCβGSI markers indicating the positions of supercoiled (sc) and nicked circular (nc) DNA (M) and linear (lin) DNA (M'); lanes 1-6, DNA rescued from samples (lanes 1-6) above.

This paper is dedicated to the memory of James Leadbetter.

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Figure 6. Effect of 9-d embryonic RCE on β globin transcript levels. Primer extension analysis (top). Lane M, molecular weight marker, 3' end-labeled pAT153/HpaII digest showing fragment sizes 92, 78, and 69 ntds; (lanes 1-3), RNA from a single oocyte injected with 1 ng pCβGSI (lane 1), plus 40 ng adult RCE (lane 2), plus 40 ng 9-d RCE (lane 3); (lane 4) RNA from a single oocyte injected with 1 ng pAT153 and 40 ng 9-d RCE; (lane 5) 5 ng poly(A+) RNA from chicken reticulocytes. The position of the β globin cap sites is indicated. Southern injection control (bottom). Lanes M and M', uninjected pCOGSI markers showing the positions of supercoiled (sc) and nicked circular (nc) DNA; lanes N, N', N'', uninjected pAT153 controls showing supercoiled (sc), nicked circular (nc), and linear (lin) DNA; lanes J-4, DNA rescued from samples (lanes J-4) above. The filter was probed with nick-translated pAT153.
References

Bendig, M. M., and J. G. Williams. 1984. Fidelity of transcription of Xenopus laevis globin genes injected into Xenopus laevis oocytes and unfertilised eggs. Mol. Cell. Biol. 4:2109-2119.

Cameron, I. L., and D. M. Prescott. 1963. RNA and protein metabolism in the maturation of the nucleated chicken erythrocyte. Exp. Cell Res. 30:609-612.

Davies, H. G., 1961. Structure in nucleated erythrocytes. J. Biochem. Biophys. Cytol. 7:671-687.

Dingwall, C., and R. A. Laskey. 1986. Protein import into the cell nucleus. Annu. Rev. Cell. Biol. 2:367-390.

Dumont, J. N. 1972. Oogenesis in Xenopus laevis (Daudin). J. Morphol. 136:158-180.

Emerson, B. M., and G. Felsenfeld. 1984. Specific factor conferring nuclease hypersensitivity at the 5' end of the chicken adult β globin gene. Proc. Natl. Acad. Sci. USA. 81:95-99.

Emerson, B. M., C. D. Lewis, and G. Felsenfeld. 1985. Interaction of specific nuclear factors with the nuclease-hypersensitive region of the chicken adult β globin gene: nature of the binding domain. Cell. 41:21-30.

Emerson, B. M., J. M. Nickel, and T. C. Fong. 1989. Erythroid-specific activation and derepression of the chick β globin promoter in vitro. Cell. 57:1189-1200.

Feinberg, A. P., and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to a high specific activity. Anal. Biochem. 137:266-267.

Galliard, L. K., K. P. Foley, Z. Yang, and J. D. Engel. 1989. The β globin stage-selector element factor is erythroid-specific promoter/enhancer binding protein NF-E4. Genes Dev. 3:1845-1859.

Gargiulo, G., and A. Worcel. 1983. Analysis of the chromatin assembled in germlinal vesicles of Xenopus oocytes. J. Mol. Biol. 170:699-722.

Gurdon, J. B., and D. A. Melton. 1981. Gene transfer in amphibian eggs and oocytes. Ann. Rev. Genet. 15:189-218.

Gurdon, J. B., and M. P. Wickens. 1983. The use of Xenopus oocytes for the expression of cloned genes. Methods Enzymol. 101:370-386.

Harland, R. M., H. Weintraub, and S. L. McKnight. 1983. Transcription of DNA injected into Xenopus oocytes is influenced by template topology. Nature (Lond.). 302:38-43.

Jackson, P. D., T. Evans, J. M. Nickol, and G. Felsenfeld. 1989. Developmental modulation of protein binding to β globin gene regulatory sites within chicken erythrocyte nuclei. Genes Dev. 3:1860-1873.

Jones, N. C., J. D. Richter, D. L. Weeks, and L. D. Smith. 1983. Regulation of adenovirus transcription by an Ela gene in microinjected Xenopus laevis oocytes. Mol. Cell. Biol. 3:2131-2142.

Kernell, A. M., L. Bolund, and N. R. Ringertz. 1971. Chromatin changes during erythropoiesis. Exp. Cell Res. 65:1-6.

Landes, G. M., B. Villeponteau, T. M. Pribyl, and H. G. Martinsson. 1982. Hemoglobin switching in chickens. J. Biol. Chem. 257:11008-11014.

Leonard, M. W. 1989. The regulation of Xenopus laevis β globin gene expression: associated changes in chromatin structure and trans-acting factor-DNA interactions. Ph.D. thesis. University of London, London. 83-84.

Lewis, C. D., S. P. Clark, G. Felsenfeld, and H. J. Gould. 1988. An erythrocyte-specific protein that binds to the poly(dG) region of the chicken β globin gene promoter. Gene Dev. 2:863-873.

McLcad, W. J., N. McLean, and Y. A. Baynes. 1972. RNA synthesis in chicken erythrocytes. Nat. New Biol. 238:137-139.

Marsh, P. J. 1989. The expression of human immunoglobulin E epsilon chain fragments in E. Coli. Ph.D. thesis. University of London, London. 57-70.

McGhee, J. D., W. I. Wood, M. Dolan, J. D. Engel, and G. Felsenfeld. 1981. A 200 base pair region at the 5' end of the chicken adult β globin gene is accessible to nuclease digestion. Cell. 27:45-55.

McKnight, G. A. 1977. A colorimetric method for the determination of sub-microgram quantities of protein. Anal. Biochem. 78:86-92.

McLean, N., and W. J. Madgwick. 1973. The RNA of chicken erythrocytes. Cell Differ. 2:271-278.

Miller, T. J., and J. E. Mertz. 1982. Template structural requirements for transcription in vivo by RNA polymerase II. Mol. Cell. Biol. 2:1595-1607.

Mohun, T., N. Garret, and J. B. Gurdon. 1986. Upstream sequences required for tissue-specific activation of the cardiac actin gene in Xenopus laevis embryos. EMBO (Eur. Mol. Biol. Organ.) J. 5:3185-3193.

Old, R. W., G. E. Sweeney, and A. R. Brooks. 1988. Activity of a cloned Xenopus albumin gene promoter in the homologous frog oocyte system. Biochem. Biophys. Acta. 951:220-225.

Partridge, A. G., N. J. Yarwood, and T. R. Rutherford. 1984. Human globin gene transcription in injected Xenopus oocytes: enhancement by sodium butyrate. EMBO (Eur. Mol. Biol. Organ.) J. 3:2787-2792.

Plumb, M. A. R. H. Nicholas, C. A. Wright, and G. H. Goodwin. 1985. Multiple sequence-specific DNA binding activities are eluted from chicken nuclei at low ionic strengths. Nucleic Acids Res. 13:4047-4055.

Ruberti, I., and A. Worcel. 1986. Mechanism of chromatin assembly in Xenopus oocytes. J. Mol. Biol. 189:451-476.

Ruiz-Carillo, A., L. J. Waugh, C. Littman, and V. G. Allfrey. 1974. Changes in histone acetyl content and in nuclear non-histone protein composition of avian erythroid cells at different stages of maturation. J. Biol. Chem. 249:7358-7368.

Ryogi, M., and A. Worcel. 1984. Chromatin assembly in Xenopus oocytes: in vivo studies. Cell. 37:21-32.

Schorpp, M., U. Dobbeling, U. Wagner, and G. U. Ryffel. 1988. β-flanking and 5'-proximal exon regions of the two Xenopus albumin genes. Deletion analysis of constitutive promoter function. J. Mol. Biol. 199:83-93.

Walmsley, M. E., and R. K. Patient. 1987. Highly efficient ß globin transcription in the absence of both a viral enhancer and erythroid factors. Development. 101:815-827.

Weintraub, H. 1985. Assembly and propagation of repressed and derepressed chromosomal states. Cell. 42:705-711.

Williams, J. G., and P. J. Mason. 1985. Hybridisation in the analysis of RNA. In Nucleic Acid Hybridisation, A Practical Approach. E. D. Hames, and S. J. Higgins, editors. IRL Press Limited, Eynsham, Oxon, England. 152-159.

Zentgraf, H., U. Scheer, and W. W. Franke. 1975. Characterisation and localisation of the RNA synthesised in mature avian erythrocytes. Exp. Cell Res. 96:81-95.