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**EXPRESSION OF HISTONE H5 mRNA DURING
ERYTHROPOIESIS IN *XENOPUS LAEVIS***

by

PETER G. FOSTER, B.Sc. (Hons.)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

Master of Science

Department of Biology
Carleton University
Ottawa, Ontario

March, 1990

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ERYTHROPOIESIS IN *XENOPUS LAEVIS*"**

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Thesis co-supervisor

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April, 1990

ABSTRACT

Steady-state levels of replication-independent histone H5, α -globin, and replication-dependent histone H4 mRNAs were examined in anaemia-induced erythropoiesis in *Xenopus laevis*. H5 and α -globin mRNAs were present in all erythroid cell types, with peak levels found in early orthochromatic erythrocytes. Relative H4 mRNA levels were highest in early polychromatic erythrocytes, consistent with the high rate of cell division in these cells. The length of the polyadenylated H5 message was estimated to be 2.1-2.2 kb, twice as long as avian H5 mRNA, and similar in length to the mRNAs of the mammalian homologue, H1^o. Primer extension analysis showed that the H5 mRNA has an unusually long, 1.25 kb 5' untranslated region. Transcription assays in isolated nuclei showed that α -globin was transcribed at all stages of recovery from anaemia, but that little H5 transcription was detectable, and this only in early stages. This, together with persistence of H5 mRNA in mature erythrocytes, suggests that H5 mRNA is very stable.

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Abbreviations

AEV	avian erythroblastosis virus
b, bp	base, basepair
CCS	1 mM sodium citrate, 1 mM CDTA, 0.1% SDS, pH 6.8
CDTA	<i>trans</i> -1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EPPS	N-[2-hydroxyethyl]-piperazine-N'-3-propanesulfonic acid
H1, H5, etc.	histones 1, 5, etc.
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	isopropylthio- β -galactoside
kb, kbp	kilobase, kilobasepair
KGB	potassium glutamate buffer: 2x KGB is 200 mM potassium glutamate, 50 mM tris-acetate (pH 7.6), 20 mM magnesium acetate, 100 μ g/ bovine serum albumin, 1 mM 2-mercaptoethanol
MEL	murine erythroleukemia
MMH	methylmercuric hydroxide
MS-222	tricaine methanesulfonate
NaOAc	sodium acetate
NRC	National Research Council of Canada
NSB	25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 5 mM DTT, 50 mM tris-HCl pH 8.0
nt	nucleotide
PIPES	piperazine-N-N'-bis[2-ethanesulfonic acid]
RES	0.5 M LiCl, 1 M urea, 0.25% SDS, 20 mM sodium citrate, 2.5 mM CDTA, pH 6.8
RNase	ribonuclease
RF	replicative, double-stranded, form of M13

RSB	10 mM tris-HCl (pH 7.4), 10 mM NaCl, 3 mM magnesium chloride
SDS	sodium dodecyl sulphate
SSC	0.15 M NaCl, 0.015 M sodium citrate, pH 7.0
TB	terrific broth: 1.2% (w/v) Bacto Tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 0.017 M KH_2PO_4 , 0.072 M K_2HPO_4
TBE	89 mM tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0
TE	10 mM tris-HCl, 1 mM EDTA, pH 8.0
TES	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
tris	2-amino-2(hydroxymethyl)aminomethane
UTR	untranslated region of mRNA located at 5' and 3' ends

I. INTRODUCTION

A. Erythropoiesis

The development of vertebrate red blood cells, erythropoiesis, has been studied extensively both as a model of terminal differentiation (Sinclair and Brasch, 1975) and as a model system for the study of gene expression (Harrison, 1984). Expression of the globins, the protein moieties of haemoglobin, have been especially well studied, because they are cell-specific, 'switched-on' during erythropoiesis, and made in very large amounts.

A key nuclear protein synthesized during red cell development in several erythropoietic systems is histone H5 (or its mammalian homologue, H1^{*}), and its expression at the mRNA level is the topic of this thesis. H5 and H1^{*} form a subset of the lysine-rich, H1-like, or 'linker' histones, so-called because they are found on the linker DNA of nucleosomes in chromatin (reviewed in van Holde, 1989, and Widom, 1989). To place H5 expression in context, an overview of other aspects of erythropoiesis will be presented, and then the topic of H5 will be revisited.

B. Erythropoietic systems

Generally speaking, immature stages of erythroid cell development are not easily obtained because they are not found in the circulation of adult animals in large number. To overcome this

constraint, various experimental strategies- erythropoietic systems- have been devised, some of which will be described here.

1. Embryonic development

Primitive erythropoiesis involves maturation of erythroid cells with high synchrony. This system has been exploited in the chick (for example Urban *et al.*, 1980; Sugarman *et al.*, 1983; reviewed in Weintraub, 1975), where embryos are easily accessible, and in the fetal mouse, reviewed by Fantoni *et al.* (1975). Mature red cells from adult mammals are enucleated, but primitive, or yolk-sac-derived, erythrocytes in the mouse are nucleated in maturity, undergoing progressive chromatin condensation, like non-mammalian adult erythropoiesis. An advantage of these systems is that no experimental manipulations are required to induce erythropoiesis, and so the process of differentiation is assuredly normal.

2. Anaemia-induced regeneration

Animals respond to experimentally induced anaemia by replacing lost blood cells, so immature stages of erythroid cell maturation may be obtained in this way. Anaemia can be induced by bleeding, but more often by injection of phenylhydrazine, which is toxic to red cells. Since animals are responding to stress, this strategy is one step removed from being completely normal.

However it is thought that anaemia-induced erythropoiesis is similar enough to normal erythropoiesis that parallels may validly be drawn (Grasso and Shephard, 1968). This strategy has been exploited to obtain rabbit reticulocyte lysate for *in vitro* translation of messenger RNA (Pelham and Jackson, 1976). Normal erythropoiesis in adult mammals takes place in the bone marrow, and reticulocytes, enucleated cells which still contain protein synthesis machinery, are released into the circulation to complete the final steps of terminal differentiation there. One can obtain younger cells from the bone marrow of anaemic rabbits. Phenylhydrazine-induced anaemia in mice causes a large erythropoietic response in the spleen. Normal erythropoiesis in adult birds also takes place in the bone marrow, but, in contrast to mammals, some immature cells, as early as erythroblasts, are released into the peripheral circulation (Lucas and Jamroz, 1961). In anaemia, the proportion of immature cells in the bone marrow and in the peripheral circulation greatly increases.

Induction of total anaemia in birds and mammals is fatal, but virtually total anaemia can be successfully induced in amphibians. The newt has been used for this purpose (Grasso and Shephard, 1968; Grasso 1973a; 1973b; Grasso *et al.*, 1977). Erythropoiesis in urodeles takes place in the spleen and in the peripheral circulation. Removal of the spleen limits erythropoiesis in anaemic animals to the peripheral circulation, where the cells are readily accessible. A similar system which has been more widely used is the anaemic African clawed toad, *Xenopus laevis* (Thomas and MacLean, 1975), which is the system used in this thesis. Injection of phenylhydrazine

results in complete loss of circulating red blood cells and initiation of a wave of erythropoietic cells in the circulation. Dead and damaged red cells are taken up by phagocytic cells in the liver and spleen (Chegini *et al.*, 1979). Using this system, a red cell developmental series from erythroblasts to mature cells can be easily obtained.

3. Transformed cells in culture

Murine proerythroblasts which have been infected with the Friend virus complex are transformed, and differentiation is arrested at this stage. These murine erythroleukemia (MEL), or Friend, cells can be induced to differentiate by addition of various substances to the culture medium (Marks and Rifkind, 1978). Differentiation of these cells is similar to normal erythropoiesis, and can proceed as far as enucleation (Sherton and Kabat, 1976; Volloch and Houseman, 1981; Patel and Lodish, 1987). K562, a transformed erythroid cell line from humans, can also be induced to differentiate (Andersson *et al.*, 1979).

Chicken erythroid cells transformed with the avian erythroblastosis virus (AEV cells) are arrested at the proerythroblast stage of differentiation, but temperature-sensitive mutants have been found which allow differentiation to proceed when the cells are shifted to the non-permissive temperature (Beug *et al.*, 1982). Another avian erythroblastosis virus, S13, has been used to transform proerythroblasts, and a temperature-sensitive mutant has been found that is able to differentiate (Knight *et al.*, 1988). Cell

culture systems offer many advantages over whole-animal systems, such as the ability to manipulate culture conditions, and freedom from contamination with other cell types. However, these cell lines are transformed, live in artificial environments, and may have lost erythropoietin dependence: these cells are far from normal, so extrapolation to normal cells should be guarded.

C. Gene expression in erythropoiesis

In all erythropoietic systems, the blood cells undergo several processes during terminal differentiation, the most obvious of which is haemoglobin synthesis. (Expression of globin will be discussed in section D, below.) The rate of cell division slows, and eventually stops. Other events include a decrease in RNA and DNA synthesis, which is followed by a decrease in protein synthesis, making the mature red blood cell one of the most genetically inert of all cells (Madgwick *et al.*, 1972; MacLean *et al.*, 1973). Ribosomes and mitochondria are almost completely lost at maturity. During differentiation there are also marked changes in the cytoskeleton and cell surface (Lazarides, 1987; Gambino *et al.*, 1984; Patel and Lodish, 1987), in nuclei and the nuclear matrix (Ringertz and Bolund, 1974; Long *et al.*, 1979), and in enzymes of intermediary metabolism and haem synthesis (Denton *et al.*, 1975; Grandchamp *et al.*, 1985).

These changes can be viewed as changes in gene expression. To express a protein, the gene must be transcribed, the transcript processed to form mRNA, which is transported to the cytoplasm and

translated, perhaps repeatedly, into a polypeptide, which folds to comprise all or part of a protein. Control points for regulation of expression, where these processes are facilitated or inhibited, may take place at any of these stages.

Probably the most important control point is the regulation of gene transcription. The vast majority of genes in a cell are not expressed. Active chromatin- *i.e.*, chromatin containing genes which are currently, recently, or potentially transcribable- is preferentially sensitive to nucleases, and this general nuclease sensitivity (as distinguished from hypersensitivity) appears to be due to 'high-mobility group' proteins HMG14 & 17 (reviewed by Weisbrod, 1982). Globin chromatin in nuclei of chick red cells is preferentially digestible with nucleases. This can be reversed by removal of HMG14 & 17, and restored by reconstitution with HMG14 & 17. Although it seems that nuclease sensitivity of active chromatin is dependent on HMG14 & 17, it is not shown why these proteins bind differently to different chromatins. In HMG14 & 17-depleted chromatin, some unknown difference must remain between active and inactive chromatin which these proteins can discern, but which nucleases cannot. Polynucleosomes from micrococcal nuclease digested chromatin which are soluble in 0.15 M NaCl contain active genes (Ridsdale *et al.*, 1988). These polynucleosomes contain less linker histone than bulk chromatin, and reconstitution experiments show that they have an altered association with linker histones. Electrophoretic fractionation of DNA from micrococcal nuclease digested chicken red cell nuclei followed by Southern blot analysis

has indicated that active chromatin also has an altered nucleosome structure, and does not generate nucleosomal oligomeric 'ladders' as does inactive chromatin (Delcuve *et al.*, 1988).

Since active chromatin is preferentially sensitive to DNase I, nick translation of isolated nuclei using biotinylated nucleotides preferentially labels active chromatin in intact nuclei, which can be visualized by fluorescence microscopy (Hutchison and Weintraub, 1985). When this was done with chick or newt red cell nuclei, active chromatin was found in interchromatinic channels, which communicate with the nuclear periphery, bordering chromatin masses. In order to show which specific genes were labelled, this was repeated using ^{32}P as label, the DNA was isolated, and used to probe Southern blots. Genes known to be expressed in these cell lineages, including histones, histone H5, and α -globin, were labelled, but several inactive genes, including ovalbumin and vitellogenin, were not. These results are consistent with the autoradiographic localization of labelled uridine in interchromatinic channels of nuclei of mature chicken erythrocytes (Zentgraf *et al.*, 1975).

Regulation of transcript processing or transport are other control points: abortive degradation of new transcripts is common (Imaizumi-Scherrer *et al.*, 1982; Therwath *et al.*, 1984). Selective translational control of different mRNA species has been shown upon induction of MEL cells (Yenofsky *et al.*, 1983). For example, the messages for p40 and p20, although present and stable (Krowczynska *et al.*, 1985) after induction, are not translated.

Messenger RNA can, of course, be translated repeatedly, until the message is degraded. Thus another important control mechanism, one which the cell exploits extensively, is differential regulation of the stability of different mRNA species (reviewed by Brawerman, 1987; 1989; Bernstein and Ross, 1989). Most messenger RNAs are polyadenylated, and in most cases the poly(A) tract is added shortly after a conserved AAUAAA sequence in the 3' UTR (untranslated region) of the message (Birnstiel *et al.*, 1985).

Although mRNA stability has often been correlated with the presence of a poly(A) tail on the message (Ruderman *et al.*, 1979; Peltz *et al.*, 1987; Bernstein and Ross, 1989), other sequences in the 3' UTR of the message are important (Shaw and Kamen, 1986). In MEL cells, at least, there is no correlation between polyadenylation and stability of several messages (Krowczynska *et al.*, 1985).

As visualized using one-dimensional fluorograms, peripheral rabbit reticulocytes and mature red cells from *X. laevis* each synthesize at least 30 non-globin proteins (Thiele *et al.*, 1979; Winning and Browder, 1988). This synthesis in *X. laevis* is actinomycin D- insensitive, and rabbit reticulocytes have no nuclei, implying that the mRNAs involved are stable. Parker and Housman (1985), using 2-D gels, were able to resolve about 330 different proteins from Friend cells, and showed that when the cells were induced, about half of them changed synthesis rate, turnover rate, or modification. So there is dramatic rearrangement in the protein complement of differentiating red cells, which slows, but does not cease, as the cells mature.

Complexity of mRNA populations in erythroid cells has been studied using hybridization kinetics (Imaizumi-Scherrer *et al.*, 1982). In late stage duck erythroblasts there are about 200 species of polysomal mRNA, but there are about 1200 other cytoplasmic mRNA species which exist as translationally-repressed mRNP particles (including the message for the poly(A) binding protein). Furthermore, the number of different kinds of transcripts which become exported from the nucleus was shown to be a small fraction of all transcripts, indicating regulation at the level of transcript processing or transport.

Brief mention will be given here to some specific examples of gene expression in erythropoiesis. Globin and histone expression will be discussed in sections D and E.

The nuclear nonhistone proteins are a complex group of proteins about which little is known (van Holde, 1989), but a few examples of changes in expression can be given here. Topoisomerase II, which cleaves and rejoins both strands of double-stranded DNA, appears to be a marker for cell proliferation (Heck and Earnshaw, 1986). The amount of topoisomerase II in chick erythroid nuclei drops precipitously after the cells have made their final cell division. The related enzyme topoisomerase I, which cleaves and rejoins one strand of double-stranded DNA, has been found in mature chicken red cells (Hoepfner and Muller, 1985), but it is not known if its activity changes during differentiation. Amounts of the high mobility group proteins HMG1 & 2 are greatly reduced when MEL

cells differentiate (Seyedin *et al.*, 1981). Nuclear nonhistone proteins have been studied in erythroid cells from *X. laevis* by electrophoretic characterization (Hilder *et al.*, 1975). Many quantitative changes, including an overall threefold decrease in the nonhistone protein to DNA ratio, but few qualitative differences, were found as the cells matured.

Proto-oncogenes are normal, cellular homologues of viral transforming oncogenes, and are thought to be important in cellular processes such as proliferation, differentiation, and signal transduction. Expression of proto-oncogenes has been studied in erythropoietic cell culture systems. In normal MEL cells, there is a transitory drop in expression of *c-myc* mRNA in the first hour after induction, perhaps the first gene expression change after induction (Ramsey *et al.*, 1986). This is due to a decrease in both transcription of the gene and stability of the message (Mecchti *et al.*, 1986). A non-polysomal-associated cytosolic factor which degrades *c-myc* mRNA has been found to be sensitive to nuclease, suggesting a nucleic acid component (Brewer and Ross, 1989). The amount of *c-myc* protein parallels the amount of *c-myc* message (Richon *et al.*, 1987). In MEL cells which have a transfected, constitutively expressed human *c-myc* gene, differentiation is blocked (Dmitrovsky *et al.*, 1986). In these cells, expression of proto-oncogenes (endogenous *c-myc*, *c-myb*, *c-fos*, *c-K-ras*, and p53), and housekeeping genes (ornithine decarboxylase, histone H3, and glyceraldehyde phosphate dehydrogenase) was affected little by constitutive *c-myc* expression (Coppola *et al.*, 1989). Induction of

MEL also causes an increase in *c-fos* and a decrease in *c-myb* mRNA levels (Ramsay *et al.*, 1986). Artificial up-regulation of a *c-myb* transgene blocks differentiation in these cells (McClinton *et al.*, 1990). The avian erythroblastosis virus has two viral oncogenes: *v-erbA* and *v-erbB*. The cellular homologue of *v-erbA*, *c-erbA*, encodes the receptor for triiodothyronine. It is transcribed in erythroid cells, with highest amounts found in late erythroblasts (Hentzen *et al.*, 1987).

Chicken reticulocytes transcribe heat shock protein HSP70 mRNA, but it is not translated unless the cells are heat-shocked (Bannerji *et al.*, 1984). However in *X. laevis*, in the absence of heat shock, an HSP-70-like protein accumulates in orthochromatic erythrocytes (Winning and Browder, 1988).

The transition from reticulocytes, which use oxidative phosphorylation to generate ATP, to mature erythrocytes, which are strictly glycolytic, involves nearly complete loss of mitochondria (Hunt, 1989). Loss of mitochondria is attributable to an erythroid-specific lipoygenase, which lyses mitochondria by peroxidation. The enzyme activity, and the protein, is found only in older reticulocytes, and not in bone marrow cells, young reticulocytes, or mature red cells. So its expression must be controlled at the translational level: the mRNA must be sequestered, then activated sometime after enucleation (Thiele *et al.*, 1979). Lipoygenase mRNA from young peripheral reticulocytes is found in a translationally inactive mRNP form, but the message, after deproteinization, is translationally active (Thiele *et al.*, 1982). The cDNA for RBC lipoygenase has been cloned

and sequenced (Fleming *et al.*, 1989). It has an intriguing 19-mer sequence repeated ten times in the 3' UTR, which may be involved in its translational regulation. The enzyme is encoded by the most abundant mRNA after α - and β -globin, and the protein can be plainly seen as a major Coomassie-stained band in a gel of reticulocyte lysate (Hunt, 1989).

D. Globin gene expression

Globin is the major protein synthesized by *X. laevis* erythroid cells at all stages of differentiation from erythroblasts to mature erythrocytes (Winning and Browder, 1988). Transcription of globin occurs in erythroid cells from the erythroblast stage onward. Transcription of globin can be detected in isolated nuclei from mature cells in the chicken (Affolter *et al.* 1987) and in *X. laevis* (Tata and Baker, 1985; Winning and Browder, 1988), although this transcription is found at very low levels, consistent with the general decrease in RNA synthesis. Among uninduced cultured cells, little or no globin mRNA is detectable (Krowczynska *et al.*, 1985; Therwath *et al.*, 1984; Knight *et al.*, 1988), but the amount of globin mRNA greatly increases upon induction (Sheffery *et al.*, 1984; Profous-Juchelka *et al.*, 1983). Chromatin changes, as defined by nucleases, around globin genes have been reviewed by Harrison (1984). DNA methylation inversely correlates with gene expression for a few genes, including β -globin. This correlation is not universal, however, and exceptions exist (Bird, 1984). Choi and Engel (1986), by deletion

mutation and transfection, examined the tissue-specificity and temporal expression of the chicken β -globin gene, and an erythroid-specific enhancer, 0.4 kb downstream from the polyadenylation site, was defined (see also Hesse *et al.*, 1986).

Although it is controversial (see Discussion), the usual primary transcript of globins is considered to be a 15s pre-mRNA containing the two introns. The mature 9s mRNA is formed by removal of the two introns and truncation of the 3' end prior to polyadenylation (Harrison, 1984). In *X. laevis*, the mature α -globin mRNA is about 0.7 kb (Kay *et al.*, 1980). Absolute rates of transcription and mRNA formation in MEL cells *in vivo* have been measured, and it has been shown that most transcripts were processed to mRNA (Ganguly and Skoultchi, 1985). However, uninduced AEV-transformed cells don't have detectable amounts of globin mRNA in the cytoplasm, but long, 7-8 kb transcripts of α -globin have been detected in the nuclei (Therwath *et al.*, 1984). The β^A -globin gene in mature chicken erythrocyte nuclei is transcribed at about half the rate of this gene in immature erythroid nuclei, yet the cytoplasm of mature chicken red cells contains no detectable globin mRNA (Affolter *et al.*, 1987). In both of these examples, the transcripts are abortively metabolized, and are not converted to mature mRNA, indicating some regulation at the level of transcript processing.

Although globin expression is regulated mainly at the transcriptional level, there is some translational control. In duck reticulocytes, most globin mRNA is polyribosomal (Imaizumi-

Scherrer *et al.*, 1982), but some, 10-20%, can be isolated in mRNP particles which cannot be translated, although they can be translated after stripping the protein (Civelli *et al.*, 1980). Another interesting translational control of globin synthesis has been shown in primitive murine erythropoiesis (Fantoni *et al.*, 1975). When mitosis is inhibited by irradiation prior to the final cell division, the population gives rise to half the control number of cells, but each cell makes twice the control amounts of haemoglobin. Thus it appears that the population is programmed to make a specific amount of haemoglobin, even if replication is inhibited.

In Friend cells, globin mRNA has a half-life of greater than 60 hours, and is about as stable as rRNA (Volloch and Houseman, 1981; Krowczynska *et al.*, 1985). The extreme stability of this message is underlined by the observation that its half-life greatly exceeds the cell-cycle time of these cells in the first few days after induction of differentiation. The cell-cycle time increases several days after induction (Brown *et al.*, 1985).

E. Histone gene expression

1. Replication-dependent histones

Cell-cycle regulation of histone genes has been reviewed by Schumperli (1986) and by Marzluff and Pandey (1988). When cells exit S-phase, or if DNA synthesis is inhibited, replication-dependent histone mRNA amounts rapidly decrease due to both a decrease in

their transcription and an increase in their degradation (Sittman *et al.*, 1983; Heintz *et al.*, 1983). However, histone mRNAs are stabilized by translation inhibitors, with or without DNA synthesis inhibition (Stimac *et al.*, 1984).

The cell-cycle transcriptional regulatory region of the hamster H3 gene has been localized to the 5' end of the gene (Artichevsky *et al.*, 1985). Using a cell-free transcription system, the decrease in transcription of rat H3 and H4 genes observed in non-dividing cells was shown to be due to 5' promotor sequences of the genes and to factors present in nuclear extracts (Larson *et al.*, 1989). Dalton and Wells (1988a) have identified a 7-nucleotide promotor element, the 'H1-box' from chicken H1 genes, which confers cell-cycle control of transcription on these genes. They also showed that this element interacts with a *trans*-acting factor, H1-SF. The amount of this *trans*-acting factor parallels the transcription rate of H1 genes in AEV cells (Dalton and Wells, 1988b). Gallinari *et al.*, (1989) have identified two H1 promotor binding factors from HeLa cells, one of which appears to be the human H1-SF homologue.

Several studies have shown that regulated degradation of replication-dependent histone mRNAs depends on the correct positioning of the conserved stem-loop structure in their 3' UTR (Levine *et al.* 1987; Capasso *et al.*, 1987; Graves *et al.* 1987). Indeed, when a construct was made such that the last 30 nucleotides of the mouse H3 mRNA was put on the end of a human α -globin gene, it was degraded like histone mRNA upon inhibition of DNA synthesis (Pandey and Marzluff, 1987). However, Morris *et al.* (1986) found

that sequences that destabilize the human H3 mRNA were located in the 5' UTR, so the importance of 3' UTR sequences for degradation is apparently not universal.

The nuclease which degrades H4 mRNA is associated with ribosomes (Graves *et al.* 1987; Peltz and Ross, 1987) but can be dissociated from them (Ross *et al.*, 1987). In their cell-free *in vitro* assay system for mRNA decay, Peltz and Ross (1987) showed that H4 mRNA contained in K562 cell polysomes was degraded more rapidly if a cytoplasmic S130 post-polysomal fraction and histones were added. Therefore histone synthesis appears to be autoregulated, like tubulin synthesis (Cleveland, 1988), by end-product inhibition. This mechanism provides a reasonable explanation for both mRNA degradation on DNA synthesis inhibition, and mRNA stabilization on protein synthesis inhibition.

2. Replication-independent histones

a. H5 and H1^{*}

The archetype of this class of histone is the erythrocyte-specific avian H5 (Neelin and Butler, 1961; Neelin *et al.*, 1964). The mammalian homologue H1^{*} is found in various differentiated tissues (Panyim and Chalkley, 1969). A search of H1^{*}/H5-like proteins has been made in eukaryotes (Smith *et al.*, 1984), and they appear to be found only in vertebrates. A preliminary study of *X. laevis* histones suggested the presence of an erythrocyte-specific H5-like histone

(Destrée, 1975). However, subsequent studies have demonstrated that this 'H5', like H1^{*}, was found in many adult tissues (Risley and Eckhardt, 1981; Brown *et al.*, 1981; Moorman and de Boer, 1985; Moorman *et al.*, 1987). Since this histone was neither erythrocyte-specific (therefore not H5) nor mammalian (therefore not H1^{*}), it has been variously called H1E (Risley and Eckhardt, 1981), H1^s (Brown *et al.*, 1981; Rutledge *et al.*, 1984) the H5 variant (Moorman *et al.*, 1984), or the H1^{*}/H5 variant (Moorman and de Boer, 1985; Moorman *et al.*, 1987). Rutledge (1987) renamed this histone 'H5' to emphasize that it is part of a subclass distinct from H1. It is called H5 in the present thesis, its lack of red cell specificity notwithstanding.

H5 and H1^{*} are expressed in several erythropoietic systems, but see section F, below, for exceptions. H5 adds to, but does not entirely replace, H1 in the differentiation of red cells (Urban *et al.*, 1980; Affolter *et al.* 1987). In uninduced AEV cells the H5 gene is transcribed at low levels, and the protein is present in small amounts, which makes H5 an earlier differentiation marker than globin in these cells (Dalton *et al.*, 1986a; Affolter *et al.* 1987). Using immunofluorescence, Banchev *et al.* (1988) showed that Friend cell H1^{*} is localized mainly in nucleoli of uninduced cells, but it is localized mainly in the periphery of the nucleus in induced cells.

The content and incorporation of phosphate into avian H5 in immature cells is higher than that in mature cells, pointing to progressive dephosphorylation of this histone associated with chromatin condensation (Adams *et al.*, 1970; Gasaryan *et al.*, 1978; Seligy and Neelin, 1973; Tobin and Seligy, 1975; Sung, 1977). In an

in vitro transcription system, dephosphorylated H5 makes a much better repressor of transcription than does phosphorylated H5 (Gasaryan *et al.*, 1978). Therefore dephosphorylation of H5, rather than the presence of the protein as such, may cause genome inactivation.

The chicken H5 gene has been sequenced. It contains no introns, and is unlinked to other histones (Krieg *et al.*, 1983; Ruiz-Carrillo *et al.*, 1983). The duck H5 gene (Doenecke and Tonjes, 1984) has been used to clone a human H1^{*} gene (Doenecke and Tonjes, 1986), which has in turn been used to find the mouse H1^{*} cDNA and gene (Alonso *et al.*, 1988; Breuer *et al.*, 1989).

By synchronizing uninduced AEV cells to various points in the cell cycle, it was shown that transcription of H5 is constitutive throughout the cell cycle, but that other histone genes were transcribed essentially only in S-phase (Dalton *et al.*, 1986a). Significant amounts of H5 transcription could be detected in mature chicken red cells, although the mature message could not be detected in the cytoplasm of these cells (Affolter *et al.* 1987). Cheng and Skoultschi (1989) cloned a cDNA for H1^{*}, and showed that in MEL cells expression of this message is induced very early in differentiation, well before the large increase in globin mRNA. H1^{*} transcription is apparently negatively regulated by *c-myc*.

Nuclease sensitivity of chromatin in the vicinity of the chicken H5 gene has been examined (Renaud and Ruiz-Carrillo, 1986). There are several hypersensitive sites near the gene, but these sites change only slightly during maturation of red cells.

Nuclear matrix association may be required for H5 transcription (Dalton *et al.*, 1986b).

The flanking regions of the chicken H5 gene have been examined for regulatory sequences, and several have been defined, both tissue-specific and non-tissue-specific (Trainor *et al.*, 1987; Rousseau *et al.*, 1989; Wigley and Wells, 1987, but note that there are inconsistencies among these studies). One example is an enhancer found in the 3' flanking region of the gene. Because this element is homologous to the globin 3' enhancer, coordinate regulation of these erythroid-specific genes in chicken red blood cells is proposed (Trainor *et al.*, 1987; Rousseau *et al.*, 1989; Trainor and Engel, 1989). The promoter of the mouse H1^{*} gene has been analysed, showing that only 5' sequences were responsible for its expression in differentiated mouse F9 cells (Breuer *et al.*, 1989). An erythroid-specific enhancer would not be expected in this gene.

The H5 mRNA from the chicken was the first of this group to be characterized. It is polyadenylated (Molgaard *et al.*, 1980), but lacks the conserved putative polyadenylation signal AAUAAA in the 3' UTR. Instead, there is a UAUAAA sequence, which may function as the poly(A) addition signal (Affolter and Ruiz-Carrillo, 1986). Particular transcription stop points apparently do not exist for H5 in the chicken, and so there is no unique H5 pre-mRNA species (Affolter and Ruiz-Carrillo, 1986). Several other H5 and H1^{*} messages have been characterized, and a few tentative generalizations emerge:

1) all are polyadenylated, but not all have the conserved AAUAAA polyadenylation signal, 2) avian H5 mRNAs are short, about 1 kb, while mammalian H1⁺ messages are more than twice this length, 3) the 3' UTRs of H1⁺ mRNAs are very long, greater than 1 kb, and 4) the 5' UTRs of these mRNAs range from about 0.1 kb in avian H5 to about 0.3 kb in mammalian H1⁺ (Affolter and Ruiz-Carillo, 1986; Doenecke and Tonjes, 1984; Kress *et al.* 1986; Alonso *et al.*, 1988; Breuer *et al.*, 1989). The *X. laevis* H5 mRNA has been shown to have a 0.2 kb 3' UTR, like avian H5 mRNAs, but its 5' UTR is >0.4 kb, unlike any other known message in this class of histone (Rutledge *et al.*, 1988). The stability of none of these messages is known.

b. Other replication-independent histones

The murine H1 var.1 gene makes two mRNAs in MEL cells: a short, cell-cycle regulated, poly(A)⁻ mRNA which ends just after the conserved stem-loop sequence, and a long, replication-independent message which is polyadenylated after a downstream AAUAAA sequence. The poly(A)⁺ message is found in higher amounts in differentiating tissues, and it is apparently negatively regulated by *c-myc* in MEL cells in a manner very similar to H1⁺ regulation in these cells (Cheng and Skoultchi, 1989; Cheng *et al.*, 1989). Two chicken H1 genes have recently been shown to differentially express both poly(A)⁺ and poly(A)⁻ messages, but their expression in erythroid cells has not been studied (Kirsh *et al.*, 1989).

The two H3.3 genes in the chicken code for replication-independent histones, with intron-containing genes, and polyadenylated mRNA (Brush *et al.*, 1985). Although the two genes code for identical proteins, the nucleotide sequences of the two genes have diverged widely in the introns, untranslated regions, and in the third position of codons. These genes are expressed in developing red cells (Brown *et al.*, 1985). The chicken H3.3B cDNA is polyadenylated 13 nt 3' to an AUUAAA sequence, even though there is an A AUAAA sequence, 0.2 kb upstream, within the 3' UTR (Dodgson *et al.*, 1987)

The H2A_F gene of chicken contains introns, and neither its transcription nor its mRNA stability is S-phase regulated in AEV cells (Dalton *et al.*, 1989). Its message is polyadenylated, and has a GAUAAA sequence instead of AAUAAA at the appropriate place relative to the site of polyadenylation. It is expressed in AEV cells, but it is not known if there is any correlation of its expression with differentiation.

E. The function of histones H5 and H1*

This class of histone has a structural role in chromatin, similar to that of H1, involved in folding of the 10 nm nucleosome filament into the 30 nm chromatin filament (reviewed in Widom, 1989). Historically, study of its function *in vivo* has been necessarily limited to descriptive studies, which have correlated the presence of H5 with restriction of DNA and RNA synthesis (Sinclair and Brasch 1975;

Ringerz *et al.*, 1985), and of H1* with terminal differentiation (Panyim and Chalkley, 1969; Osborne and Chabanas, 1984). However, in order to distinguish cause from effect, one must be able to have the amount of H5 as the sole experimental variable, which has been technically difficult to do. A preliminary study addressed the possible role of linker histones in differentiation: when H1, H5, or H1* were microinjected into uninduced Friend cells, no change in haemoglobin synthesis was found (Furusowa *et al.*, 1980). More recently, Bergman *et al.* (1988) microinjected L6 rat myoblasts with chicken H5 (or with chicken H1, lysozyme, or protamine as controls), which caused compaction of the nucleus, a decrease in the volume of the cytoplasm, and a decrease in uridine and thymidine incorporation. In another study, the chicken H5 gene, under the control of a glucocorticoid promotor, was transfected into rat sarcoma cells and induced to synthesize H5, leading to an accumulation of large amounts of H5 in these cells (Sun *et al.*, 1989). This accumulation was shown to accompany a marked decrease in cell proliferation, and a two-fold reduction in general transcription, as measured by ³H-UTP incorporation in permeabilized cells. Specific gene expression, as measured by Northern blot analysis, was measured for several genes, including those for histone, β -actin and vimentin, and *c-myc*. Some large changes were noted after expression of the H5 transgene, but it was not clear whether these changes were cause or effect of the decrease in proliferation.

In many instances, terminal differentiation takes place without H5 or H1*. Recall that in *X. laevis*, H5 is found in many

differentiated tissues, but its presence does not correlate in a simple way with the state of differentiation of these tissues (Moorman *et al.*, 1987). Although it has been controversial, it appears that there is no H1*-like protein in any cell type of chicken, and that H5 is found only in erythroid cells (Shannon *et al.*, 1985). Also, an H5 or H1*-like histone protein is not detectable in salamanders (Brown, 1982).

No cell-specific, H5-like histone has been found in nucleated erythroid cells of anaemic rabbit bone marrow (Adams and Neelin, 1976). Using an anti-mouse H1* antibody, Eisen *et al.*, (1980) examined the occurrence of H1* in the mouse and in MEL cells. It was found in induced MEL cells, some terminally differentiated tissues of the mouse, but not in erythroid cells of the normal spleen or normal bone marrow. Lennox (1986) looked at H1 and H1* presence and synthesis in splenic erythroblasts of the anaemic mouse. There was complex H1 expression, but little H1*. Apparently normal and anaemia-induced murine erythropoiesis differ in this respect from erythropoiesis in cultured, transformed, Friend cells.

Thus the H5/H1* class of histone, far from being universal, is found only in some differentiated tissues of some higher vertebrates. Functions proposed for this class of histone, such as nuclear inactivation, and induction or maintenance of differentiation, must in many cases be done by the cell through other means. As potential candidates, H1 histones are differentially regulated (van Holde, 1989), or even cell type-specific (LaBella *et al.*, 1988), and may have an important role in general repression or differential expression of the genome (Cole, 1984). Evidence has accumulated that H1 histones

play a direct role in gene repression (Schlissel and Brown, 1984; Wolffe, 1989; Weintraub, 1984).

Since there are so many genes in a genome to be repressed, it is not likely that a specific mechanism exists for each gene. More likely there is a general repressor, and linker histones appear to be the best candidates (Weintraub, 1985). With respect to this putative function, H5 appears to be more effective than H1 (Seligy and Neelin, 1970).

F. Thesis objectives

Expression of H5 at the protein level has been studied in developing red cells of *X. laevis* (Rutledge *et al.*, 1984). Although thymidine incorporation and core and H1 histone synthesis virtually cease before about day 20 of recovery from anaemia, H5 synthesis continues beyond that. A study of histone gene expression at the mRNA level in erythropoiesis in *X. laevis* was done in a Ph.D. thesis by Koster (1987). H1 and core histone mRNAs, as measured by *in vitro* translation and Northern blot analysis, decreased greatly after day 17, becoming undetectable after day 21, while H5 mRNA decreased greatly only after day 21, and was detectable up to day 26. She also speculated that the H5 mRNA was about 12s in size (0.8-1.2 kb) and was not polyadenylated. However, measurement of H5 mRNA amounts was confounded by lack of either a nucleic acid probe to detect the H5 mRNA itself, or an antibody to detect H5 made

from H5 mRNA by *in vitro* translation. Working with these constraints, Koster's attempt to clone the H5 cDNA failed.

However, during the same period, Rutledge *et al.* (1988) succeeded in cloning cDNA for two H5 variants, using an expression vector and an immunological detection method for *E. coli* expressed product. Preliminary characterization of the H5 mRNA through analysis of Northern blots of oligo(dT)-cellulose fractionated RNA (Rutledge, 1987), and the nucleotide sequence of several cDNA clones (Rutledge *et al.*, 1988), suggested that the H5 mRNA was at least 1.4 kb in size and was polyadenylated at the 3' end. The presence of dA tracts of up to 15 nucleotides in length produced by use of oligo(dT)₁₈₋₂₀ as a primer for cDNA synthesis is consistent with this latter observation.

Since only preliminary knowledge of H5 mRNA expression in developing red blood cells of *X. laevis* has been produced from previous work, I made use of the availability of the H5 cDNA clones to detail several aspects of this problem. Specific objectives for this thesis were: 1) to induce an erythroid developmental series in *X. laevis*, and to characterize these cells with respect to cell type and RNA content, 2) to measure relative steady-state levels of H5, α -globin, and H4 mRNAs by Northern blot analysis using specific cloned probes, 3) to measure the size of the H5 mRNA and the size of its 5' UTR, and 4) to determine the specific transcriptional capacity of nuclei of cells from this developmental series, thereby permitting an estimate of mRNA stability.

II. MATERIALS AND METHODS

A. Preparation of *X. laevis* red blood cells

Adult females of the South African clawed toad *Xenopus laevis*, 65-105 g, were obtained from Xenopus I, Ann Arbor, Michigan, USA. The toads were kept in dechlorinated water in a large tank which was held at 18°C with a combination of an immersible refrigeration coil and an immersible heater, both thermostatically controlled. The toads were fed Purina Trout Chow (large size) *ad libitum*.

Toads were made anaemic according to the procedure of Thomas and MacLean (1975) by intraperitoneal injections of 0.5 ml of a 5% (w/v) solution of phenylhydrazine in amphibian Ringers (Hamburger, 1942. NaCl: 6.5 g/l, KCl: 0.14 g/l, CaCl₂: 0.12 g/l, NaHCO₃: 0.1 g/l). Toads were injected twice, one day apart, the second injection being 'day 0' of recovery from anaemia.

For the collection of red blood cells, animals were anaesthetized by immersion in 500 ml 0.2% (w/v) MS222 (Sigma) in 18°C tap water for 45 minutes. The weight of the toad was noted before exsanguination. The ventral chest cavity was exposed and blood was collected by severing blood vessels near the heart and immediately diluting the blood into culture medium with heparin (see below). Collection and storage of blood was done at room temperature. Collected blood cells were stored in a shallow layer in a beaker to allow exchange of gasses. When all the blood was obtained

(about 30 minutes), cells were collected by centrifugation (IEC clinical centrifuge, full speed, 2 minutes), the volume of the pellet noted, and the cells were resuspended to 10 ml in medium. The cells were uniformly suspended and an aliquot was removed to estimate the cell number using a haemocytometer. The remainder of the cells was immediately placed in a shallow layer in a beaker until used further.

Red blood cell culture medium was Medium 199 with Hank's salts (Gibco) diluted to 0.6x, buffered with 5 mM HEPES, 2.5 mM EPPS, and 2.5 mM TES (Eagle, 1971), 0.35 g/l NaHCO_3 , pH 7.8 which was then stirred vigorously to allow the CO_2 to equilibrate with the air. After a final adjustment to pH 7.8, the medium was supplemented with antibiotic-antimycotic (0.01 volumes of a 100x concentrated solution from Gibco) and 10% (v/v) fetal bovine serum, and then sterile-filtered. For exsanguination, heparin was added to 0.2 mg/ml. 'Red blood cell saline' is this medium without serum.

For some toads, liver tissue was also collected and immediately placed on ice. One-half gram of liver was cut into small pieces and placed into ice-cold medium with heparin to allow blood to leach out from the tissue.

B. Preparation of blood smears

Cells were fixed by adding 0.25 volumes of 10% (w/v) glutaraldehyde to a suspension of cells in medium. Fixation was allowed to proceed for 10 minutes at room temperature, after which

the cells were washed in saline, and resuspended in saline. To the suspension of fixed cells was added 0.02 volumes of cytological adhesive (2% (w/v) polyvinyl alcohol, 0.2% (w/v) hydrolysed vinyl triethoxysilane, prepared as described in Fink, 1987), and appropriate amounts of the cell suspension were dried on glass microscope slides for one hour at 50-65°C. Cell smears were stained in undiluted methanolic Giemsa (BDH) for 15 minutes followed by immersion for five seconds, with agitation, in methanol. Stained cells were air dried on a slide warmer, and, while still warm, a coverslip was applied over the cells using 15 µl of Permount (Fisher).

C. RNA isolation and characterization

1. Purification of total RNA

RNA was isolated by a modified combination of two methods as described by Chomczynski and Sacchi (1987), and Birnboim (1988). The former procedure requires a stock guanidinium isothiocyanate solution containing 50 g of guanidinium isothiocyanate, 3.52 ml of 0.75 M sodium citrate (brought to pH 7.0 with 0.75 M citric acid), and 5.28 ml of 5% (w/v) sarcosyl dissolved in 58.6 ml water with warming. Solution D is made from this by adding 0.36 ml of β -mercaptoethanol to 50 ml of stock solution.

A blood cell pellet of from 0.15-1.5 ml or, in some cases, 0.5 g of minced liver was homogenized in 4 ml of solution D in a dounce homogenizer. To this was added 0.4 ml of saturated NaOAc (brought

to pH 4 with acetic acid), 4 ml of water-saturated phenol, and 0.8 ml chloroform-isoamyl alcohol (24:1), with thorough mixing after the addition of each reagent. After the addition of all the components, the suspension was vortexed well and cooled on ice for 15 minutes, after which it was centrifuged (SS-34 rotor, 10,000 rpm) for 20 minutes at 4°C. After centrifugation, RNA was present in the upper aqueous phase, and DNA and protein were present in the thick white interphase and lower organic layer (Chomczynski and Sacchi 1987). The upper layer was removed and the interphase and organic layers back-extracted with 1 ml of solution D, 0.1 ml of NaOAc solution, and 0.2 ml of chloroform-isoamyl alcohol (24:1) as above. After re-cooling and re-centrifugation as above, the two aqueous layers were combined and mixed with one volume of isopropanol overnight at -20°C to precipitate the RNA.

Precipitated RNA was centrifuged (SS-34 rotor, 10,000 rpm, 20 minutes), and the pellet dissolved in 2 ml of RES (Birnboim, 1988: 0.5 M LiCl, 1 M urea, 0.25% (w/v) SDS, 20 mM sodium citrate, 2.5 mM CDTA, final pH 6.8) in a 15 ml Corex tube. Proteinase K (100 µl of a 1 mg/ml aqueous solution) was added and the mixture was incubated at 50°C for 30 minutes. RNA was precipitated by addition of 167 µl of 2 M NaOAc (pH 5.2) and 5 ml of 95% (v/v) ethanol, overnight at -20°C. After centrifugation (SS-34 rotor, 10,000 rpm, 20 minutes), the pellet was dissolved in 0.9 ml RES and extracted with 0.1 ml of chloroform-isoamyl alcohol (24:1). After recovery of the aqueous phase, high molecular weight RNA was precipitated by addition of 7.5 µl of 2 M acetic acid and 1 ml of LiCl/ethanol

(3 volumes of 5 M lithium chloride plus 2 volumes of 95% (v/v) ethanol) and holding at 0°C overnight. Precipitated RNA was recovered by centrifugation (10 minutes), and the supernatant was removed. RNA was dissolved in 0.2 ml CCS (Birnboim 1988: 1 mM sodium citrate, 1 mM CDTA, 0.1% (w/v) SDS, pH 6.8) and precipitated by addition of 10 µl of 2 M sodium acetate pH 5.2 and 0.5 ml 95% (v/v) ethanol, followed by incubation overnight at -20°C, and centrifugation (30 minutes, 4°C). The CCS solubilization and ethanol precipitation step was repeated once more. The final pellet was washed in 75% (v/v) ethanol and dried before it was dissolved in water and the concentration measured spectrophotometrically. RNA, either dissolved in water or ethanol precipitated, was stored at -70°C.

2. Fractionation of RNA by oligo(dT)-cellulose chromatography

Enrichment of RNA containing polyadenylated tracts at their 3' ends, referred to as poly(A)⁺ RNA, was done essentially as described by Aviv and Leder (1972). Oligo(dT)-cellulose powder (0.2 g, Boehringer-Mannheim) was suspended in 3 ml elution buffer (10 mM tris-HCl, pH 7.5, 1 mM EDTA, 0.1% (w/v) SDS) and loaded into a small column, giving about 0.7 ml column volume. The elution buffer was drained and the matrix washed with 5 ml of 0.1 M NaOH, 2x with 5 ml elution buffer, and 3x with 5 ml of binding buffer (elution buffer with 0.5 M NaCl). RNA was diluted to 1 ml in water, and 1 ml of 2x binding buffer was added. This was incubated at 65°C for 5 minutes and then placed on ice until use. The RNA was added

to the column and the eluate collected and run through the column again. The eluate from this second passage is the poly(A)⁻ fraction. The matrix with bound RNA was washed three times with 5 ml of binding buffer. The absorption at 260 nm of the eluate was monitored to show that unbound RNA was washed away. Bound RNA was eluted with elution buffer, by passing three 1 ml aliquots through the column. As measured spectrophotometrically (A_{260}), the poly(A)⁺-enriched RNA was found in the first two fractions collected. RNA was recovered by adding one-tenth volume of 3 M NaOAc and 2.5 volumes of 95% (v/v) ethanol, overnight precipitation at -20°C, centrifugation for 30 minutes in the cold, 75% (v/v) ethanol wash, and drying of the pellet *in vacuo*.

3. Fractionation of RNA by gel electrophoresis.

Total RNA and RNA fractionated by oligo(dT)-cellulose chromatography were further fractionated by gel electrophoresis in agarose gels using one of two denaturants. Electrophoresis using formaldehyde as a denaturant was performed essentially as described by Fourney *et al.* (1988). Manipulations involving formaldehyde were done in a fume hood. Ethidium bromide was either added to the sample as described by Fourney *et al.* (1988), or, in later experiments, it was added to the molten gel, after cooling to 50°C, to a final concentration of 0.3 µg/ml. Appropriate amounts of RNA dissolved in water were dried down in microfuge tubes. An RNA ladder, 4 µl/track, was included as a reference sample,

consisting of RNA markers of sizes 9.5, 7.5, 4.4, 2.4, 1.4, and 0.24 kb, obtained from BRL. Sample buffer was freshly prepared and 20 μ l added to the dried RNA, which was then heated for 10 minutes at 65°C. The samples were cooled and loaded immediately.

Electrophoresis was performed at 25 V for 16 hours.

Electrophoresis using glyoxal as an RNA denaturant was performed as described here. Solid glyoxal trimeric dihydrate (141 mg, Sigma) was dissolved to 1 ml (2 M) in 20 mM sodium phosphate pH 7.0 by heating in a boiling water bath for two minutes with occasional vortexing. This was used to make 1 M glyoxal, 10 mM sodium phosphate pH 7.0, 50% (v/v) DMSO. RNA was denatured in 20 μ l of this solution for ten minutes at 70°C. Agarose was dissolved in running buffer, 10 mM sodium phosphate, pH 7.0, and allowed to cool to about 65°C. Sodium iodoacetate was then added to 8 mM, SDS was added to 0.1% (w/v), and the gel was cast (Skopp and Lane, 1988). Electrophoresis was at 45 mA (about 23 V), with buffer recirculation. After electrophoresis, the glyoxal-RNA complex was reversed by soaking the gel in 50 mM NaOH for one hour as described by Villarreal *et al.* (1979), followed by neutralization and staining in 0.2 M tris-HCl pH 7.0, 1 μ g/ml ethidium bromide for 45 minutes.

D. DNA probes

1. Description of clones used

Various cloned DNAs were used as DNA-RNA hybridization probes for Northern blot analysis. All except pCH3dR8 were provided by V.L. Seligy (NRC). The plasmid pCH3dR8 was a gift from A. Ruiz-Carrillo (Cancer Research Centre, Laval University School of Medicine, L'Hôtel-Dieu du Quebec). These clones are described briefly here (for more details, see Appendix 1).

a) XLH5A-1 (Rutledge *et al.*, 1988). This clone is a partial cDNA of the histone H5 gene from *Xenopus laevis*. It is a 313 bp insert in the *HincII* site of M13mp9, and consists solely of coding region sequence, from about alanine 11 to lysine 110 of H5. The clone actually used in this study was XLH5A-1X, a reversal of the insert of this clone in M13mp8. These two clones were confirmed by partially sequencing them.

b) pXG6C1 (Kay *et al.*, 1980, 1983) is an α -globin cDNA from *Xenopus laevis*. It is a 0.64 kb fragment cloned into the *PstI* site of pBR322. It was confirmed by restriction endonuclease digestion.

c) pGC42 (Childs *et al.*, 1979) is a 1.1 kb *HhaI* fragment spanning the sea urchin *Strongylocentrotus purpuratus* H4 gene, flanked by *HindIII* linkers, and cloned into the *HindIII* site of pBR322. It was confirmed by restriction endonuclease digestion.

d) pCH3dR8 (Sugarman *et al.*, 1983) is a 1.21 kbp fragment spanning the chicken histone H4 gene, cloned into the *EcoRI* site of pBR322. It was confirmed by restriction endonuclease digestion.

2. Plasmid DNA isolation

Small-scale plasmid DNA preparations ('mini-preps') were done according to Morelle (1989) except that cultures were grown in 1.5 ml TB (see section II.E.2) at 37°C. RNase digestion was not required. Large-scale plasmid preparations were done using the alkaline SDS lysis and cesium chloride centrifugation method described by Maniatis and coworkers (1982).

3. Isolation of DNA fragments

Restriction endonuclease digestion of plasmid or M13mp8/9 RF DNA was done according to Hanish and McClelland (1988) or Maniatis *et al.*, (1982). In all cases the full-length insert was excised for use as probe. DNA was fractionated on agarose-TBE gels. After ethidium bromide staining of the agarose gel, bands were cut out and the DNA recovered using the IBI electroelution apparatus according to the manufacturer's instructions. Eluted DNA was dissolved in TE buffer and the concentration was determined by gel electrophoresis and ethidium bromide fluorescence, using DNA standards of known size and amount.

4. Labelling of DNA fragments

Isolated DNA fragments (25 ng) were labelled by the random primer method of Feinberg and Vogelstein (1983) using the manufacturer's protocol (Boehringer Mannheim), except that the probe and primer DNA supplied as part of a kit were boiled together. [α - 32 P] dATP, 800 Ci/mmol, was used as the label. Unincorporated labelled dATP was removed using a spun column as described by Maniatis *et al.* (1982).

E. Bacteriological techniques

1. Bacterial strains used for propagation of cloned DNA

Various derivatives of *Escherichia coli* strain K-12 were used in the course of this work. These are: DH5 α F' (BRL), HB101, RR1, and JM103 (Miller, 1987), and JM101 (Yanisch-Perron *et al.*, 1985)

2. Media and antibiotics

Media ingredients for bacterial cell culture were from Difco. For bacterial plates, agar was added to media to 1.5% (w/v). B-agar was used for plates containing IPTG and Blueo-gal (see below), and consists of Bactotryptone (10 g), NaCl (8 g), and yeast extract (3 g) per litre of medium (modified from Messing, 1983). L-broth was used for routine bacterial culture and consists of Bactotryptone

(10 g), yeast extract (5 g), NaCl (0.5 g), 4 ml of 1 M NaOH solution, and glucose (2 g) per litre of medium (modified from Miller, 1987). TB (terrific broth) was made according to Tartof and Hobbs (1987), and was used for 'mini-preps' (see above, section II.C.2) and for high-yield cultures. Minimal medium M9 (Messing, 1983, plus histidine, proline, and leucine) was used for propagation of JM101 and JM103 cells. To detect β -galactosidase activity, a combination of IPTG and Blueo-gal was used. A 100 mM solution of IPTG (BRL), an artificial inducer of the *Lac* operon, was made by dissolving 23.8 mg in 1 ml of water, and sterile-filtering the solution. A 2% (w/v) solution of the chromogenic substrate for β -galactosidase 'Blueo-gal' (BRL) was made by dissolving 20 mg in 1 ml of dimethylformamide. B-agar was autoclaved and allowed to cool to 55°C before addition of 10 μ l of inducer and 50 μ l of substrate per 25 ml of medium, the amount used for one petri plate. A stock solution of ampicillin was made in water at 3 mg/ml and sterile filtered, and used at 30 (for pBR322) to 75-150 (for pUC) μ g/ml final. Tetracycline stock solution was made in ethanol at 1.5 mg/ml, and used at 15 μ g/ml final (Maniatis *et al.*, 1982). Kanamycin stock was 2.5 mg/ml in water, sterile-filtered, and used at 70 μ g/ml (Viera and Messing, 1987).

3. Transformation of bacteria

Three methods were used to make 'competent' *E. coli* and then to transform them with DNA. The calcium chloride method (Maniatis *et al.*, 1982) was used with frozen competent HB101 cells

provided by P. Shwed (NRC). The method of Hanahan (1984) with DH5 α F' was used for viral DNA. For routine transformations, the method of Chung *et al.* (1989) was used, with JM103 cells.

F. Northern blot analysis

1. Electroblotting

Blotting of gel fractionated RNA to a nylon membrane (Hybond-N) was done by electrotransfer using a Hoefer 'Transphor' apparatus. The transfer buffer was 12 mM tris-HCl, 6 mM NaOAc, 0.3 mM EDTA, pH 7.5 following protocols provided by New England Nuclear (1985). Electroblotting was done at 10 V for two hours at 5°C and then at 40 V for two hours at 5°C. The RNA on the membrane, still damp, was fixed under UV light for two minutes (Khanjian, 1986). In some cases ethidium bromide fluorescence on the membranes was photographed during fixation. The membranes were never allowed to dry out completely.

2. Hybridization and autoradiography

Prehybridization, hybridization, and washes were done at 65°C for homologous probes, and at 42° or 50°C for heterologous probes. Hybridization and prehybridization solution consisted of 1 M NaCl, 1% (w/v) SDS, and 100 μ g/ml denatured salmon sperm DNA. Prehybridization was done in 50 ml for at least 30 minutes.

The blot was then placed in a 'seal-a-meal' bag together with 4 ml of fresh, pre-warmed hybridization fluid. Labelled probe was denatured in a boiling water bath for ten minutes and then added to the bag, which was then sealed, and hybridization allowed to take place for 18-24 hours. Blots were washed three times for 5 minutes at room temperature, then twice for 30 minutes at the hybridization temperature, and then ten minutes at room temperature, in 2x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1 % (w/v) SDS. Blots were then covered in plastic film and exposed to Kodak RP or AR film at -70°C using an intensifying screen.

3. Removal of probes from membranes

To strip blots of probe in preparation for another hybridization, blots were placed in boiling 0.1 % (w/v) SDS, which was then allowed to cool slowly to room temperature.

4. Quantitation of autoradiograms by densitometry

Autoradiograms were quantitated using a Molecular Dynamics computing densitometer, Model 300A. The average density of a line around the perimeter of the autoradiographic bands was used as the baseline for area integration of the interior of the line. Density readings for series of bands were normalized to the strongest band, with no correction for photographic non-linearity.

G. *In vitro* transcription in isolated nuclei.

1. Preparation of nuclei

A suspension of 2×10^8 cells was pelleted, the supernatant removed, and, on ice, the cells were resuspended in cold RSB (Groudine *et al.* 1981, 10 mM tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl_2) containing 0.02% (w/v) Nonidet P-40. The 0.5% concentration of NP-40 suggested by Groudine *et al.* (1981) for avian erythroid cells was found to be too high, and caused nuclear lysis in *X. laevis* red cells. The suspension was allowed to stand on ice for two minutes and then centrifuged. This step usually resulted in incomplete cell lysis, and the process was repeated at least two times more, resulting in a white pellet of nuclei. Finally the nuclear pellet was suspended in NSB (Marzluff and Huang, 1984: 25% (w/v) glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, 5 mM DTT, 50 mM tris-HCl pH 8.0) and frozen at -70°C .

2. Transcription assay

Frozen nuclei (section II.G.1) were thawed on ice, centrifuged, and resuspended to about 120 μl in NSB. A 2x concentrated transcription solution, 100 μl per assay, was 1 mM ATP, CTP, and GTP, 0.05 mM S-adenosyl methionine, 240 mM KCl, and 5 mM magnesium acetate (Marzluff and Huang, 1984). In the first experiment, the 2x transcription solution contained 100 μCi

[α - 32 P]UTP (800 Ci/mmol) per 100 μ l and 0.1 mM unlabelled UTP, whereas in the second experiment 125 μ Ci [α - 32 P]UTP were used, with no unlabelled UTP. The reaction was started by adding 100 μ l of nuclear suspension to 100 μ l of 2x transcription solution, allowing the mixture to stand at 0°C for one minute to mix, and then transferring to 18°C for 30 minutes (first experiment) or to 20°C for 45 minutes (second experiment) to allow transcription to take place. An aliquot of 10 μ l from the remaining 20 μ l was diluted in NSB and the number of nuclei counted using a haemocytometer.

3. Isolation of *in vitro*-transcribed RNA

In vitro-synthesized RNA was isolated in a manner similar to that described in section II.C.1. The reaction was stopped by mixing in 1.3 ml of Chomczynski and Sacchi solution D. To this was added sequentially, with agitation between each addition, 150 μ l of saturated sodium acetate pH 4.0, 1.5 ml phenol, and 300 μ l chloroform-isoamyl alcohol (24:1). This was shaken vigorously for 10 seconds and then cooled on ice for 15 minutes. After centrifugation (10,000x g, 20 minutes, at 4°C), the aqueous upper layer was removed and RNA precipitated with one volume of isopropanol at -20°C overnight. The RNA precipitate was collected by centrifugation (10,000x g, 20 minutes, at 4°C) and dissolved in 0.5 ml RES (section II.C.1), before addition of proteinase K (1 mg/ml, 25 μ l), incubation of the mixture at 50°C for 30 minutes, and extraction with

an equal volume of chloroform-isoamyl alcohol (24:1). The RNA was then precipitated by addition of 42 μ l of 2 M NaOAc pH 5.2 and 1.42 ml of 95% (v/v) ethanol and allowed to stand at -70°C for 15 minutes and at -20°C for 15 minutes. The purified RNA was collected by centrifugation (10,000x g, 20 minutes, at 4°C), and washed in 75% (v/v) ethanol.

4. Measurement of specific *in vitro*-transcribed RNA by hybridization

In order to assay specific RNA transcripts, nylon membranes containing 0.1 μ g of DNA sequences of H5 and α -globin, and pBR322, pUC119, and M13 as controls, were prepared according to Reed and Mann (1985). The miniature blots were prehybridized in 1 M NaCl, 1% (w/v) SDS, 100 μ g/ml denatured salmon sperm DNA, 50 μ g/ml yeast tRNA for the first experiment, and in the same solution with 50% (v/v) formamide for the second experiment at 65°C (first experiment) or 42°C (second experiment).

The radiolabelled RNA was dissolved in 2 ml (first experiment) or 0.5 ml (second experiment) of hybridization solution, which was the same as the prehybridization solution described above. Small samples (10 μ l for the first experiment, 5 μ l for the second experiment) were taken for scintillation counting. For the second experiment, 10,000 cpm were removed at this point and analysed on a formaldehyde gel (section II.C.3). Hybridization with the remainder of the RNA took place in individual hybridization bags

for 24 hours (first experiment) or 72 hours (second experiment). Blots were then washed as described in section II.F.2 at 65°C and exposed to Kodak AR film.

H. Primer extension analysis of H5 mRNA

Estimation of the length of the 5' end of the H5 mRNA was done by primer extension according to Calzone *et al.* (1987) using the 29-mer oligonucleotide 5-D24R, 5'-CAA-TAT-CAT-GTC-AGA-GTA-CTT-AGG-GTG-GT-3' (V.L. Seligy, W. Sung, and M. Dove, NRC). The primer was end-labelled using polynucleotide kinase (Pharmacia) and [γ -³²P]ATP (3000 Ci/mmol) as phosphate donor. Methylmercuric hydroxide (MMH) denaturation of RNA was done by dissolving 10 µg RNA in 10 µl of 10 mM MMH and incubating at room temperature for ten minutes (Krug and Berger, 1987). The RNA-MMH solution was then treated with 1 µl of 0.35 M β-mercaptoethanol for five minutes at room temperature followed by ethanol precipitation. Labelled primer (50,000 cpm, 40 fmol) was mixed with 10 µg RNA in 5 µl of 40 mM PIPES pH 6.4, 0.4 M NaCl, 1 mM EDTA. Template RNA was total *Saccharomyces cerevisiae* RNA from K. Prakash (NRC), *X. laevis* poly(A)⁺ RNA (see section II.C.2), and *X. laevis* poly(A)⁺ RNA which had been treated with MMH. Hybridization was accomplished by heating the mixture to 70°C for three minutes, and then allowing it to cool to 40°C over half an hour.

Primer extension was done in 50 µl by addition to the hybridization mixture of 10 µl 5x cDNA buffer (5x is 500 mM tris-HCl

pH 8.3, 50 mM MgCl₂, 250 mM KCl), deoxyribonucleotides to 1 mM, 25 U human placental ribonuclease inhibitor (Amersham), 29 U avian myeloblastosis virus reverse transcriptase (Life Sciences) and incubating at 40°C for one hour. RNA was then hydrolysed by addition of NaOH to 50 mM and heating to 65°C for one hour. The solution was then neutralized by addition of 20 µl tris-HCl pH 7.4 and 2.6 µl 1 M HCl, and then the DNA precipitated by addition of 0.1 volumes 3 M NaOAc (pH 5.2) and 2 volumes of ethanol. After recovery of the DNA by centrifugation, it was resolved on a denaturing alkaline agarose gel (Maniatis *et al.*, 1982). Molecular weight markers were derived from phage λ DNA digested with *Hind*III and *Eco*RI, and then end-labelled using T4 DNA polymerase (IBI) and [α-³²P] dATP (800 Ci/mmol) as described by Maniatis *et al.* (1982). After electrophoresis, the gel was fixed in 7% (w/v) TCA for one half hour and then compressed overnight between paper towels, covered with a glass plate and a weight. The gel, about 1 mm thick after compression, was placed in a plastic bag and exposed to Kodak AR film with an intensifying screen for one day at -70°C.

1. Subcloning of H5 cDNA

The clone XLH5A-2 is a cDNA of histone H5, which starts at the threonine-8 codon and runs to the end of the 3' UTR, together with a few bases of the poly(A) tail (Rutledge *et al.*, 1988). The 777 bp insert in the *Hinc*II site of M13mp9 was confirmed by sequencing part of it. The insert in XLH5A-2 was subcloned into the

phagemid vector pUC119 (Viera and Messing, 1987) as described here. The RF form of XLH5A-2 was digested (section II.D.6) with *HindIII* and *EcoRI* and the 0.78 kbp fragment isolated. The ends were filled-in using the large fragment of *E. coli* DNA polymerase I. The fragment (26 ng) was blunt-end ligated into *HincII*-digested pUC119 (75 ng) in 0.5x KGB buffer (2x KGB is 200 mM potassium glutamate, 50 mM tris-acetate (pH 7.6), 20 mM magnesium acetate, 100 µg/ml bovine serum albumin, 1 mM 2-mercaptoethanol) described by Hanish and McClelland (1988), 0.5 mM ATP, and 1 U T4 DNA ligase (BRL) in 20 µl overnight at room temperature.

Ligated DNA (2 µl) was used to transform JM103 cells according to the procedure of Chung *et al.* (1989), and a transformant was found with the *PvuII* site of the insert near the *EcoRI* site of the vector. Orientation was confirmed by restriction analysis, and the cloning site near the *EcoRI* site was confirmed by sequencing a single-stranded preparation of the clone using the dideoxy chain termination method (Sanger *et al.*, 1977), the '-40' primer (New England Biolabs), [α -³⁵S]dATP, and modified T7 DNA polymerase (Sequenase, United States Biochemical Corporation) according to the manufacturer's instructions for sequencing near the primer. This clone was called MV2, and was used in the DNA blots for 'run-on' transcription (II.G.4).

III. RESULTS

A Characterization of erythropoiesis

1. Induction of anaemia

Adult toads were made anaemic by injection with phenylhydrazine and allowed to recover for various times before collection of blood cells. Two series of toads were injected. In the first series, individual toads were allowed to recover for 10, 15, 20 and 25 days before sacrificing, while in the second series these same days were used plus days 30, 40, and 60. Both series included untreated toads as controls. Since erythropoiesis in anaemic toads takes place in the peripheral circulation (Thomas and MacLean, 1975), this system allows access to staged points in the latter parts of red cell development (Rutledge *et al.*, 1984).

2. Microscopic characterization of regenerating erythrocytes

Blood smears from anaemic toads obtained after each recovery time point and from control toads were prepared and stained with Giemsa as described in section II.B. Counts of the different kinds of cells were made, based on cell morphology and staining criteria described by Thomas and MacLean (1975), and by Lucas and Jamros (1961) (Table 1).

Table 1. Differential Cell Counts^a

DAY	ME ^b	OE	PE	EB	D/M	WC
First series						
10	1.6	-	81.5	0.3	3.3	13.3
15	0.6	-	84.9	-	11.5	2.9
20	-	96.9 ^c	-	-	2.3	0.8
25	-	95.1	0.2	-	0.3	4.4
control	99.6	-	-	-	-	0.4
Second series						
10	16.4	-	72.6	0.2	4.8	6.0
15	0.1	-	94.5	-	3.8	1.6
20	-	94.7 ^c	3.1	-	1.0	1.1
25	-	97.8	0.6	-	0.5	1.0
30	-	97.9	0.2	-	0.2	1.7
40	-	98.2 ^d	0.5	-	0.1	1.2
60	-	98.8 ^d	0.1	-	-	1.1
control	99.3	-	-	-	-	0.7

^a Blood cells from anaemic and control toads were affixed to slides, and stained with Giemsa (as described in Materials and Methods, section II.B). The different kinds of cells were counted, which are shown here as per cent of the total in each population. Greater than one thousand cells were counted for each slide.

^b Abbreviations: ME: mature erythrocytes; OE: orthochromatic erythrocytes; PE: polychromatic erythrocytes; EB: erythroblasts; D/M: damaged and mitotic; WC: white cells.

^c OE here are late PE/early OE.

^d OE here are late OE, very similar to ME.

The earliest cells in these recovery series were from day 10 after injection with phenylhydrazine. At this time a few mature erythrocytes, many of which were misshapen or otherwise abnormal, remained. Basophilic erythroblasts were very rare (0.2-0.3%), having by this time been supplanted by polychromatic erythrocytes. At subsequent times in the recovery series, polychromatic erythrocytes changed to orthochromatic erythrocytes, and by days 40 and 60, the orthochromatic erythrocytes were very similar to mature erythrocytes. In early stages, days 10 and 15, the erythroid cells were heterogeneous in size and shape, but in late stages the cells became more uniform.

Many mitotic cells were seen in early stages. In all such cases the cells were small and damaged, unlike living mitotic cells seen by phase contrast microscopy. Presumably the collapse of these cells was an artefact of preparation. (It should not be assumed that all damaged cells are therefore mitotic. Because mitotic cells could not reliably be distinguished from merely damaged cells, the two classes were counted together (Table 1)).

A high proportion of white cells was found in early stages compared to late stage and mature populations. In these early stages, however, this is a proportion of a smaller number of cells (Table 2); thus variation expressed in absolute numbers of white cells recovered per toad is less, but still appreciable (data not shown).

Table 2. Analysis of RNA yields from regenerating blood

Day	Weight of toad (g)	Total number of cells ($\times 10^8$) ^a	RNA recovered (μ g)	Normalized total RNA (μ g) ^b	RNA/cell (μ g/ 10^7 cells)
First Series					
10	98	5.4	1218	994	23
15	77	6.8	1148	1193	17
20	74	18	1844	1994	10
25	86	18	1830	1702	10
control	92	39	123	107	0.32
Second Series					
10	78 & 93 ^c	3.76 (1.76) ^a	893	893 ^d	50.7
15	64	12.8 (4.8)	691	2303	14.4
20	76	14.8 (6.8)	1027	2353	15.1
25	105	61 (53)	2455	2153	4.6
30	69	20.5 (12.5)	433	823	3.5
40	75	35 (27)	730	1009	2.7
60	65	45 (37)	600	898	1.6
control	74	37.5 (29.5)	151	208	0.5

^a In the first series, all the cells were used for RNA extraction, but in the second series some of the cells were used to prepare nuclei. The number of cells in the second series actually used to prepare the RNA is indicated in parentheses.

^b The amount of RNA/toad has been normalized to an average 80 g toad, the average weight of all toads used.

^c In the second series, day 10, two toads were used. The number of cells recovered (3.76×10^8) is the total from both toads

^d For the second series, the total RNA/toad has been normalized both for the size of the toad and for the proportion of total cells used for the RNA preparation.

3. Cell counts and RNA yields during recovery from anaemia

At the end of each recovery period, the toad was exsanguinated as totally as practicable (section II.A). Blood from day 10 toads was transparent brown, and gave cell pellets of only about 150 μ l, while blood from later stages was the more familiar opaque red, with cell pellets of 1-1.8 ml. In order to obtain sufficient cell numbers, the blood from two toads at day 10 was combined in the second series. Data on cell counts and RNA yield from these cells are presented in Figure 1 and Table 2. Figure 1 shows that the number of cells per toad rises 10-fold from $2-5 \times 10^8$ to $4-5 \times 10^9$ during this period of recovery from phenylhydrazine-induced anaemia. There is some variability among individual toads (for example, of the toads used, the series 2, day 25 toad was the largest, and the richest source of blood cells). The total RNA, normalized for toad size, shows that both populations as a whole more than double the amount of RNA during the period from day 10 to day 25 (Table 2). However, since this RNA comes from more than three times as many cells, the amount of RNA per cell falls several-fold during the same period. In later periods of recovery (after day 25), the normalized amount of RNA in the population falls by more than half as the number of cells rises, so that RNA/cell falls toward normal levels (Figure 1). Thus, while the number of cells rises about ten-fold (day 10 to day 60), RNA/cell falls about thirty-fold, but it is still several-fold more at day 60 than in control cells (Table 2).

Figure 1. Cell counts and RNA yields

Two series of toads were made anaemic and exsanguinated at the indicated times ('Day of recovery'), and the cells were counted. (Blood cells from two toads were combined for day 10 of the second series.) Total cellular RNA was extracted and measured spectrophotometrically, and expressed here as RNA per 10^7 cells. Data is taken from Table 2.

First series **Second series**

B. General observations on steady-state mRNA levels

In order to assess the relative amounts of specific mRNA species, Northern blot analyses were done on the RNA extracted from blood samples described in Table 2. In these experiments, RNA from each of the two anaemia series was fractionated by formaldehyde gel electrophoresis using 10 μ g of total RNA in each lane (section II.C.3). After photography, using ethidium bromide fluorescence to visualize the RNA, the RNA within the gels was transferred to nylon membranes by electrophoresis. The membranes were hybridized with each of the four probes (H5, α -globin, Sp H4, and Ch H4) and exposed to X-ray film, stripping the membrane of hybridized probe between each hybridization. The results of two such experiments are shown in Figures 2 and 3. Panel A of each Figure shows mainly the presence of ethidium bromide-stained ribosomal RNA bands. The discrete rRNA 18s and 28s bands and specific bands on Northern blots (panels B, C, and D in each Figure) show that the RNA isolation gave generally intact RNA. As the cells matured, however, there was a general decrease in rRNA yield relative to total RNA. This trend is especially evident in the 28s rRNA band of the RNA from the later days of the second series. In control, mature red blood cells, the 28s rRNA band stains less intensely than the 18s rRNA band. This is probably not an artefact of RNA isolation because it is observed as part of a trend, and mRNA species, as shown in Northern blots from the same samples, are not degraded. In goose and chicken red blood

Figure 2. Northern blot analyses: first series

RNA from blood (lanes a-e) and liver (lanes g-k) was extracted from anaemic and control toads of the first series, and fractionated by formaldehyde gel electrophoresis, together with marker RNA (lane f, see section II.C.3). Recovery periods were: lanes a and g, day 10; lanes b and h, day 15; lanes c and i, day 20; lanes d and j, day 25; lanes e and k, control toads. Panel A: ethidium bromide stained gel. Panels B, C, and D are Northern blots hybridized respectively with: H5, α -globin, and sea urchin SpH4 probes.

A

B

C

D

a b c d e f g h i j k a b c d e f g h i j k a b c d e f g h i j k

Figure 3. Northern blot analyses: second series

RNA from blood of anaemic (lanes b-h) and control (lane i) toads was extracted and, together with marker RNA (lane a, see section II.C.3), fractionated by formaldehyde gel electrophoresis. Recovery periods were: lane b, day 10; lane c, day 15; lane d, day 20; lane e, day 25; lane f, day 30; lane g, day 40; lane h, day 60. Panel A: ethidium bromide stained gel. Panels B, C, and D are Northern blots hybridized respectively with: H5, α -globin, and chicken ChH4 probes. The arrow in panel A shows the ethidium bromide-stained band corresponding to the α -globin band in panel C.

A

B

C

D

a b c d e f g h i a b c d e f g h i a b c d e f g h i

cells, the ratio of 28s to 18s changes from 2 to 1.2 during maturation (V.L. Seligy, personal communication).

The corresponding Northern blots are shown in Figures 2 and 3, panels B, C, and D. Blots probed with α -globin sequence gave an adequate exposure in a matter of hours, but blots hybridized with H5 probes require about a day for adequate exposure. Since lengths and specific activities of the probes and hybridization conditions were similar, this indicates that the amount of α -globin mRNA generally exceeded that of H5 mRNA. This is consistent with differentiating chicken erythroid cells, where the amount of β^A -globin mRNA greatly exceeded the amount of H5 mRNA (Affolter *et al.*, 1987). The two probes used to detect H4 mRNA were heterologous (Appendix 2). The sea urchin *Strongylocentrotus purpuratus* SpH4 probe gave a visible exposure in a reasonable time for the first series (Figure 2, D), but not with the blot of the second series. The reverse situation was seen with the chicken ChH4 probe. In both usable blots there was considerable non-specific hybridization, especially to 28s rRNA, while 18s rRNA blocked non-specific hybridization with ChH4 probe (Figure 3, D).

The first series of Northern blots (Figure 2) shows RNA from both red cells and liver. There is very little α -globin or H5 signal in control livers (Table 3). Although as much H5 is found in toad liver as in red cells (Brown *et al.*, 1981), it appears that the amount of its mRNA in normal liver is much less than in normal red cells. Since developing erythroid cells contain much more RNA per cell than do mature red cells, the presence of relatively large amounts of H5 and

Table 3. Normalized scans of Northern blots^a

First Series

<u>Day</u>	<u>Probes for</u>					
	<u>H5</u>		<u>α-Globin</u>		<u>H4</u>	
	<u>RBC</u>	<u>liver</u>	<u>RBC</u>	<u>liver</u>	<u>RBC</u>	<u>liver</u>
10	0.146	0.0154	0.330	0.0467	1.000	0.121
15	0.258	0.0349	0.554	0.123	0.283	0.0590
20	1.000	0.0468	0.762	0.243	0.105	0.0566
25	0.525	0.0217	1.000	0.280	0.104	0.0643
control	0.912	0.00730	0.310	0.00164	0.434	0.0324

Second Series

<u>Day</u>	<u>Probes for</u>		
	<u>H5</u>	<u>α-Globin</u>	<u>H4</u>
10	0.0577	0.0669	0.471
15	0.0139	0.417	1.000
20	0.0595	1.000	0.399
25	1.000	0.977	0.113
30	0.000601	0.845	0.310
40	0.722	0.352	0.157
60	0.0591	0.269	0.315
control	0.131	0.148	0.348

^a Autoradiograms from Northern blot analyses (Figures 2 and 3) were quantitated with a computerized scanning densitometer (section II.F.4), and normalized by assigning 1.0 to the strongest band in each blot.

α -globin mRNA in livers from anaemic toads may be partly due to blood contamination (Table 3). However, ratios of the relative amounts of H5 mRNA in red cells and livers during erythropoiesis do not change in parallel with the ratios of α -globin mRNA in red cells and livers. This suggests that erythropoiesis also occurs in the liver (Chegini *et al.*, 1979), and that the relative amounts of H5 and α -globin mRNAs in this putative liver erythropoiesis differ from those in erythropoiesis in the peripheral circulation.

The Northern blot analyses of red cell RNA presented in Figures 2 and 3 show the amounts of specific mRNA (mRNA_{sp}) relative to a constant 10 μg of total RNA ($\text{mRNA}_{\text{sp}}/\text{total RNA}$). As described earlier, the amount of total RNA/cell decreases about 100-fold over the erythropoietic series studied; therefore each lane of these gels contains the RNA from different numbers of cells. A desirable descriptor of erythropoiesis would be $\text{mRNA}_{\text{sp}}/\text{cell}$, obtainable by

$$\frac{\text{mRNA}_{\text{sp}}}{\text{total RNA}} \times \frac{\text{total RNA}}{\text{cell}} = \frac{\text{mRNA}_{\text{sp}}}{\text{cell}}$$

To this end, the RNA blots were first quantitated using a computerized scanning densitometer and used to obtain $\text{mRNA}_{\text{sp}}/\text{cell}$, and then normalized by setting the maximum for each specific mRNA to 1 (Figure 4, Table 3).

The most obvious trend seen from $\text{mRNA}_{\text{sp}}/\text{cell}$ analyses is that H4 mRNA is highest in earliest stages (day 10-15), falling

Figure 4. Specific mRNA/cell

Specific mRNAs from anaemic toads were measured by Northern blot analysis (Figures 2 and 3), and the autoradiograms were quantitated by densitometry to obtain relative specific RNA per total RNA (Table 3). This was then multiplied by RNA/ cell (Table 2) to obtain relative specific mRNA per cell, as shown.

First series

■ H5
■ globin
▨ H4

Second series

■ H5
■ globin
▨ H4

rapidly to low levels about 5 days later and continuing near control levels thereafter. Trends for H5 and α -globin mRNAs are less obvious. Levels for each are high but variable at day 10, and the peak amounts for each are about day 20-25, after which the amounts of both fall gradually to very low levels in late-stage and mature cells. Small but significant amounts of H5 mRNA are present in normal cell populations. There are two anomalies in the H5 blot of the second series at days 15 and 30. Both of these points show little H5 mRNA compared to their neighbours and to the overall trend. Levels of α -globin mRNA do not show these anomalies. The ethidium bromide-stained gel shows that the RNA profile for these time points is also anomalous in that there appears to be more RNA between the two rRNA bands, which is not consistent with overall RNA degradation.

Further analysis of the Northern blots was carried out to determine heterogeneity and sizes of these mRNAs. The α -globin related sequences indicate one main component of 0.68-0.73 kb (in agreement with the size of 670 ± 50 nt as measured by Kay *et al.*, 1980), and two minor ones at 2.8 kb and >6 kb in length. The high molecular weight bands may tentatively be assigned to unprocessed or partially processed transcripts (see Discussion, section IV.E). It is notable that the higher molecular weight bands of α -globin are not present in RNA from mature red cells or from liver. Higher molecular weight α -globin bands were also detectable in the second series blot at exposures longer than the three-hour exposure shown in Figure 3, C. The main α -globin band appears to correspond to a

band in the ethidium bromide stained gel (Figure 3, panel A, arrow). It is known that the mature *X. laevis* α -globin mRNA is 581 bases long from cap site to poly(A) addition site (Stalder *et al.*, 1988). In the present study the size of 0.68-0.73 kb for the mature mRNA including the poly(A) tail implies a tail length of 100-150 bases.

Similar measurements taken for H5 indicated a single transcript of 2.1 to 2.2 kb in size (see section C, below). The two heterologous H4 probes that were used both detected a transcript between 0.40 and 0.44 kb in length. This is slightly larger than the 0.35 kb length of *X. laevis* H4 mRNA reported by Koster (1987), and the 0.40 kb length of chicken H4 mRNA reported by Sugarman *et al.* (1983). The H4 mRNA has a coding region of 306 nt (Appendix 2), which implies that the lengths of the untranslated regions of the message are short.

C. Size of H5 mRNA

As mentioned in the previous section, RNA blot analyses from formaldehyde gels (14 cm long, 1.2% agarose) indicates that the size of the H5 mRNA is 2.1-2.2 kb. This is in contrast to that reported by Rutledge (1987), who, using a glyoxal gel and ribosomal RNA markers, obtained a size of about 1.4 kb for H5 mRNA. Clarification of this disparity was the motivation for careful verification of the size of the H5 message.

To obtain higher resolution of H5 mRNA, long (25 cm) gels were used along with RNA markers (BRL ladder) as size standards.

Three kinds of gels were run: formaldehyde with 1.2 or 1.0% agarose, and glyoxal with 1.2% agarose. Northern blot analyses were done on each, using the H5 probe for detection, and the results are shown in Figure 5. Both formaldehyde gels gave estimated sizes of 2.2 kb, and the glyoxal gel gave an estimated size of 2.1 kb. Ribosomal RNA sizes were also estimated in these three gels, and all these results are compiled in Table 4. That the sizing of the rRNA, known to have much secondary structure, was in close agreement with reported values implies that the size of the H5 given here is accurate. The RNA used in these analyses was poly(A)⁺ RNA from day 25 red cells. There are two H5 mRNA species expressed in red cells- H5A and H5B (Rutledge *et al.*, 1988)- but there was no evidence in these long gels for their resolution.

D. H5 mRNA is found in the poly(A)⁺ fraction

RNA from day 25 red cells was chromatographically enriched in polyadenylated RNA by use of an oligo(dT)-cellulose column. Unfractionated RNA (10 µg), poly(A)⁻ or 'flow-through' RNA (3 and 10 µg), and poly(A)⁺ RNA (1 and 3 µg) were electrophoresed on a formaldehyde gel followed by Northern blot analysis using H5 and α-globin as probes (Figure 6). It is evident that the amount of H5 or α-globin mRNA in the 3 µg poly(A)⁺ lane is approximately the same as in the 10 µg unfractionated lane, with little found in either poly(A)⁻ lane. This indicates that the messages, both H5 and α-globin, are found almost exclusively in the poly(A)⁺ fraction of

Figure 5. Size of H5 mRNA

Electrophoresis in three different kinds of denaturing agarose gels, used to fractionate first series, day 25 poly(A)⁺ RNA for Northern blot analysis with H5 probe. Panel a: formaldehyde with 1.2% agarose; panel b: formaldehyde with 1.0 % agarose; panel c. glyoxal with 1.2% agarose. Marker RNA (see section II.C.3) was run in lanes adjacent to each and visualized, together with the rRNA bands, by fluorescence of bound ethidium bromide.

4.4
28s
2.4
18s
1.4

a

b

c

Table 4. Estimated RNA sizes^a

	formaldehyde 1.2% agarose	formaldehyde 1.0% agarose	glyoxal 1.2% agarose	reported sizes
28s rRNA	3.9	3.8	4.15	4.110 ^b
18s rRNA	2.0	1.85	1.9	1.826 ^c
15 mRNA	2.2	2.2	2.1	1.4 ^d 0.8-1.2 (12s) ^e

^a Sizes, given in kb, were estimated from 25 cm gels described in section III.C., using RNA markers described in section II.C.3.

^b Ware *et al.*, (1983)

^c Atmadja *et al.*, (1984)

^d Rutledge (1987)

^e Koster (1987)

Figure 6. Analysis of oligo(dT)-fractionated RNA

RNA from red cells of the first series, day 25-recovered toad was fractionated by oligo(dT)-cellulose chromatography and electrophoresed on a formaldehyde gel. Lane a contains 10 μ g of unfractionated RNA, lanes b and c contain 3 and 10 μ g, respectively, of poly(A)⁻ RNA, and lanes d and e contain 1 and 3 μ g, respectively, of poly(A)⁺ RNA. Panel A shows the ethidium bromide stained gel. Panels B and C show Northern blot analyses using H5 probe (panel B), or α -globin probe (panel C).

RNA, and that the two messages have both been enriched about three-fold in the poly(A)⁺ fraction. When one compares the ethidium bromide fluorescence of the 3 µg poly(A)⁻ with that of the 3 µg poly(A)⁺ lanes, it can be seen that ribosomal RNA is depleted about two-fold in the poly(A)⁺ fraction.

It was estimated above (section III.B) that the length of the poly(A) tail of α -globin was 100-150 bases long, yet α -globin and H5 fractionated in a quantitatively similar manner. This argues against the likelihood of retention of H5 mRNA on the column solely because of its A-rich sequence within the coding region (Appendix 3).

In a longer exposure (data not shown) of the α -globin blot, the unfractionated RNA lane prominently showed the two high molecular weight bands (as in Figure 2 C, described in section III.B.), which may have been unprocessed or partially processed transcripts. However, the poly(A)⁺ RNA on this blot showed, but faintly, only the >6 kb band, consistent with absence of a poly(A) tail in the longer, unprocessed, forms. Although one would have expected that the flow-through RNA would contain the large forms, the poly(A)⁻ RNA showed neither high molecular weight band. Evidence that suggests that the high molecular weight bands may be artefactual is provided by another experiment (data not shown) used to monitor integrity of the poly(A)⁺ RNA used for primer extension analysis (see below, section E). To test for RNase activity, poly(A)⁺ RNA was dissolved in primer hybridization solution, followed by Northern blot analysis using the α -globin probe. There was no evidence for degradation,

but the >6 kb band had greatly increased in this RNA relative to that in control poly(A)⁺ RNA (see Discussion, section IV.E).

E. Primer extension analysis of H5 mRNA

Since the 2.1-2.2 kb size of the H5 mRNA shown by RNA blot analysis was larger than the 1.2 kb cDNA sequence of the message obtained by Rutledge *et al.* (1988), the question is raised: Is the extra sequence at the 5' end, the 3' end, or both? To answer this, primer extension analysis was done on H5 mRNA. In this experiment, a 29-mer oligonucleotide primer was made to be complementary to the region from leu-33 to asp-24. Leu-33 begins at nucleotide 691 of the known cDNA sequence, measured from the polyadenylation site (Appendix 3).

The labelled primer was allowed to anneal to three different RNAs: yeast total RNA as a control, *X. laevis* poly(A)⁺ RNA, and *X. laevis* poly(A)⁺ RNA which had been pre-treated with methylmercuric hydroxide to destroy any secondary structure which might cause premature 'stopping' of the enzyme. After primer extension using avian reverse transcriptase, the DNA was fractionated on a denaturing alkaline agarose gel and detected by autoradiography.

The autoradiogram in Figure 7 shows no evidence for primer extension against the yeast RNA, while there was significant primer extension against both of the *X. laevis* RNA templates. The newly synthesized cDNA shows as a smear, reaching up to about 1.4 kb,

Figure 7. Primer extension analysis of H5 mRNA

Poly(A)⁺ RNA from a day 25 toad and a control was annealed to a labelled H5-specific oligonucleotide primer, which was extended using reverse transcriptase, as described in section II.H. DNA was isolated and fractionated on a denaturing agarose gel, and then detected by autoradiography. Lanes a and e are marker DNA, consisting of phage λ digested with *Eco*RI and *Hind*III, and end-labelled using polynucleotide kinase and [γ -³²P]ATP. Template RNA was: lane b, yeast total RNA; lane c, *X. laevis* poly(A)⁺ RNA; lane d, *X. laevis* poly(A)⁺ RNA which had been pre-treated with methyl mercuric hydroxide. Prominent bands of 1.35 and 0.55 kb are indicated with arrows, and minor bands, of 0.94, 0.78, and about 0.3 kb, are indicated with dots.

with several discrete bands. The most intense band is at 0.55 kb, with another major band at 1.35 kb, and minor bands at 0.94, 0.78, and at about 0.3 kb. In the lane derived from the methylmercuric hydroxide-treated RNA as template there is a general shift to higher molecular weight cDNA products after extension, especially of the band at 1.35 kb.

Focussing on the longest discrete product of primer extension, the 1.35 kb band, and recalling that the primer began at 0.69 kb, one can estimate a 2.04 kb distance from the putative poly(A) addition site to the 5' end. Comparing this to the 2.1-2.2 kb size from Northern blot analyses, it is shown that almost all of the RNA is accounted for, and furthermore that most, if not all, of the missing sequence lies at the 5' end of the message. The translation initiation codon at 0.79 kb implies a 5' UTR of 1.25 kb. The H5 mRNA likely contains a poly(A) tail, and although a length of 60-160 bases for it would satisfy the above arithmetic, the precision of the Northern blot analyses does not really allow a precise estimate of its size.

F. *In vitro* transcription in isolated nuclei

In the second anaemia series, in addition to isolating RNA, nuclei were isolated for 'run-on' transcription assays, whereby transcripts already initiated *in vivo* are allowed to incorporate highly labelled precursor nucleotides. Two experiments were completed, differing mainly in that the first experiment included unlabelled UTP

in addition to [α - 32 P]UTP, whereas the second experiment was not supplemented with additional unlabelled UTP.

After *in vitro* transcription, RNA was isolated, dissolved in hybridization solution, and an aliquot counted by liquid scintillation counting. These results, presented in Table 5, show that incorporation of radioactivity into RNA in the second experiment was 10-fold to 100-fold more than in the first experiment, because the specific activity of the nucleotide was 100-fold greater in the second experiment. In both experiments it was shown that younger cells incorporated more radioactivity than more mature cells, although even control cells incorporated significant amounts of radioactivity, as was found by Hentschel and Tata (1978) and by Winning and Browder (1988). Incorporation does not correlate with the proportion of contaminating white cell nuclei (Table 1). The much higher incorporation in the 10-day-recovered cells indicates that later transcription was not limited by availability of UTP.

In the second experiment, an aliquot of 10,000 cpm of RNA was taken from each assay and analysed on a formaldehyde-agarose gel. The autoradiogram, Figure 8, shows that in recovering toads most of the RNA synthesis is found in the 0.24 to 1.4 kb size range, with some sizes apparently even larger than 1.4 kb. Although nuclei from control cells incorporated a similar amount of precursor compared to day 60 nuclei (Table 5), more of the labelled RNA was larger than 2 kb (Figure 8, lane g, day 60 *cf.* lane h, control).

In order to determine if transcription of specific genes had taken place, labelled RNA was allowed to hybridize to H5 and

Table 5. *In vitro* transcription^a

Day	Nuclei (x10 ⁷) ^b	cpm measured (x10 ⁴) ^c	Normalized cpm (x10 ⁴) ^d
First experiment			
10	1.7	2.03	12.0
15	9.0	8.16	9.1
20	9.9	5.28	5.3
25	10.8	3.30	3.1
30	7.8	1.90	2.4
40	8.9	1.04	1.2
60	11.5	0.45	0.4
control	10.2	0.33	0.3
Second experiment			
10	2.2	239	1,100
15	17.2	84.0	48.8
20	17.6	111	63.1
25	21.1	145	68.7
30	15.9	117	73.6
40	13.7	42.3	30.9
60	13.0	8.82	6.8
control	16.1	16.6	10.3

^a *In vitro* nuclear 'run-on' transcription assays were performed and RNA isolated as described in section II.G. A sample of the isolated RNA was taken for liquid scintillation counting to measure overall incorporation of ³²P UTP into RNA.

^b the number of nuclei used in each assay.

^c (cpm minus blank) ÷ (fraction of the total used as a sample)

^d normalized for 1 x 10⁸ nuclei

Figure 8. Formaldehyde-agarose gel of *in vitro*-transcribed RNA

Aliquots of labelled RNA isolated from 'run-on' transcription assays (section II.G) were analysed by formaldehyde gel electrophoresis and detected by autoradiography. Unlabelled marker RNA (section II.C.3) was run in an adjacent lane. RNA was from: lane a, day 10; lane b, day 15; lane c, day 20; lane d, day 25; lane e, day 30; lane f, day 40; lane g, day 60; lane h, control nuclei.

α -globin probes, and to control DNA, which had been blotted onto individual nylon membranes. After washing of the blots, specific RNA was detected by autoradiography. After a twenty-day exposure, the autoradiogram from the first experiment (data not shown) showed extremely faint bands for α -globin probe for days 15, 20, and 25, and for H5 probe for day 15, but all bands were almost imperceptible. The second experiment showed clear autoradiographic bands after two days exposure (Figure 9). Transcription of α -globin was shown at all stages up to and including day 60, but it was not clear, even after a longer exposure, whether control nuclei transcribed α -globin. In contrast, transcription of H5 was detected in day 10 nuclei only. A longer exposure did not reveal H5 transcription product in later stages.

**Figure 9. Detection of specific sequences from
in vitro-transcribed RNA**

RNA from 'run-on' transcription assays was isolated and hybridized to small Southern blots containing H5 and α -globin sequences, as well as control (vector) DNA, and detected by autoradiography, as described in section II.G. RNA is from: lane A, day 10; lane B, day 30; lane C, day 60, lane D, control.

IV. DISCUSSION

Phenylhydrazine-induced anaemia in *X. laevis* has been used in this study to produce cells in various stages of erythropoiesis. As well as expressing globin, these cells also express histone H5, which may play an important role in terminal differentiation of these cells as a general gene repressor, leading to a decrease in cell proliferation. Rutledge *et al.* (1984) studied expression of H5 in *X. laevis* at the protein level. Koster (1987) attempted to examine expression of H5 mRNA, but this was constrained by lack of a satisfactory detection method.

Availability of *X. laevis* H5 cDNA clones (Rutledge *et al.*, 1988) has allowed a more careful examination of H5 mRNA expression. In the present study erythropoietic differentiation was induced in *X. laevis*, and characterized with respect to cell type and RNA content. Using this system, several aspects of H5 mRNA expression were addressed: 1) relative steady-state levels of H5, α -globin, and H4 mRNAs were measured, 2) the length of the H5 message, and the length of its 5' UTR were measured, 3) the specific transcriptional capacity of nuclei from these cells was measured to estimate the stability of the H5 mRNA. Each of these aspects will be discussed, below.

A. Erythropoietic differentiation in *X. laevis*

Anaemia was induced in two series of toads, and, after recovery periods ranging from 10 to 60 days, erythroid cells were obtained for microscopic characterization, RNA extraction and transcription studies. Together with control cells from uninjected toads, these cells provide fairly homogeneous populations of an erythroid differentiation series from polychromatic erythrocytes, through orthochromatic erythrocytes, to mature red blood cells (Table 1). Comparison of erythroid cells from the present study to cells from similar studies can be made by microscopic characterization, using cell morphology and staining characteristics. This showed that the cells obtained in the present study were similar to those obtained by others using the same erythropoietic system (Chegini *et al.*, 1979; Hentschel and Tata, 1973; Rutledge *et al.*, 1984; Koster, 1987; Winning and Browder, 1988), and therefore valid parallels among these studies may likely be drawn. However, by comparison to the present study and to the other regeneration series just mentioned, the regeneration series induced by Thomas and MacLean (1975) was slow. This is corroborated by comparing rates of *in vivo* incorporation of labelled macromolecule precursors: Rutledge *et al.* (1984) showed peak incorporation of both thymidine and lysine in the earliest stages measured (day 10), while Thomas and MacLean (1975) observed peak incorporation of thymidine at day 15, and of leucine at days 20-30. The earliest cells obtained in large number in the present study, at day 10 of recovery, were

that H5 is synthesized in mature erythrocytes, the cells should be incubated with ^{35}S -methionine, perhaps extracted with 5% perchloric acid (in which only lysine-rich histone and high mobility group proteins are soluble), and the labelled protein precipitated with anti-H5 antibody. This would be followed by electrophoresis and fluorography, comparing the electrophoretic mobility of the precipitated product of *in vivo* labelling with authentic H5.

Rutledge *et al.* (1988) chose day 8 cells as the source of RNA to make cDNA banks from which to find the cDNA for H5 because these early cells were known to be very active in H5 synthesis. Based on results from the present study (Table 3), hindsight shows that cDNA banks made from orthochromatic erythrocytes or even mature erythrocytes would likely have contained a higher proportion of H5 cDNA. The low proportion of H5 mRNA in total RNA of early stage cells may also provide an explanation for the inability of Rutledge (1987) to show H5 production by *in vitro* translation of RNA from these cells.

Globin is the major protein synthesized by *X. laevis* erythroid cells at all stages from erythroblasts to mature erythrocytes (Winning and Browder, 1988). Haemoglobin has been measured in regenerating *X. laevis* erythroid cells, and found to be only 0.5x and 0.7x normal levels at days 25 and 50 respectively (Chegini *et al.*, 1979). Synthesis of haemoglobin in cells of anaemic toads continues for a long time, and haemoglobin does not accumulate to control levels until about 140 days of recovery.

polychromatic erythrocytes. The earlier erythroblast stage found in small number by Chegini *et al.* (1979) and by Rutledge *et al.* (1984) up to day 10, was found in the present day 10 population at only 0.2-0.3%.

Preliminary studies (data not shown) confirmed the importance of the temperature at which the toads were kept on the rate of recovery from anaemia (Chegini *et al.*, 1979). Based on these experiments, all subsequent work was carried out with the toad tank maintained at a constant temperature of 18°C. This practice decreased, but did not eliminate, the variability among toads seen by Rutledge *et al.* (1984).

During recovery from anaemia in *X. laevis*, the immature erythroid cells divide and differentiate in the peripheral circulation (Thomas and MacLean, 1975). In the present study, the erythroid cells of the earliest population (day 10) numbered $2-5 \times 10^8$ per toad, which increased to about 4×10^9 per toad in late stage and mature populations (Table 2). Although the dynamics of cell division in the populations could be very complex, this increase in cell number is consistent with 3-4 divisions of each of the cells of the early population to replenish the entire complement of mature red cells. The rate of protein synthesis decreases in late erythropoiesis in MEL cells (Sherton and Kabat, 1976; Parker and Housman, 1985) and in *X. laevis* (Rutledge *et al.*, 1984; but *cf.* Thomas and Maclean, 1975). This decrease is paralleled by a decrease in the cellular content of ribosomes, shown by both a decrease in cytoplasmic basophilia, and by a decrease in measurable RNA per cell (Figure 1).

Knowing that there are about 6 kb (about 2×10^6 daltons) of RNA per ribosome (Table 4), and assuming that most of the cellular RNA is ribosomal (Figures 2A and 3A), it can be calculated that the number of ribosomes decreases from about 10^6 per cell in day 10 cells to 10^4 per cell in mature cells. This decrease can only be partially explained by dilution of existing ribosomes by cell division, and requires in addition that the rate of degradation of ribosomes exceed their rate of synthesis.

The RNA content of erythroid cells in the present study decreases from 30-50 $\mu\text{g}/10^7$ cells in day 10 cells to 0.3-0.5 $\mu\text{g}/10^7$ cells in mature cells (Table 2, Figure 1). This can be compared to the cellular DNA content of 63.5 $\mu\text{g}/10^7$ cells (Thiebaud and Fischberg, 1977). The cellular RNA content in the present study is much less than that found in more active cells such as 3T3 cells (320 $\mu\text{g}/10^7$ cells) or fibrosarcoma cells (350 $\mu\text{g}/10^7$ cells), and is low, like that found in granulocytes (5 $\mu\text{g}/10^7$ cells) (Birnboim, 1988). Uninduced MEL cells have 82 μg RNA per 10^7 cells, which decreases to 55 $\mu\text{g}/10^7$ cells 4 days after induction of differentiation (Grandchamp *et al.*, 1985). This is consistent with findings of others who have shown a 40-50% reduction in RNA content in differentiated MEL cells (Sherton and Kabat, 1976; Fraser and Curtis, 1987). Although the cellular RNA content of mature chicken red cells (0.2-0.4 $\mu\text{g}/10^7$ cells, Zentgraf *et al.*, 1975) is quite similar to that found in mature *X. laevis* red cells (0.3-0.5 $\mu\text{g}/10^7$ cells, Table 2), the RNA of the chicken erythrocyte is mostly nuclear, while the RNA of

X. laevis red cells is mostly ribosomal (Figures 2A and 3A). This is consistent with an earlier comparison wherein it was shown that mature chicken red cells contained little rRNA per total RNA while mature *X. laevis* red cells contained more, and indeed synthesized ribosomal RNA to a small extent (MacLean and Madgwick, 1973; MacLean *et al.*, 1973). However, in this same comparison, *in vivo* incorporation of labelled uridine in mature *X. laevis* red cells was shown to be about 280-fold less than that in mature chicken red cells.

Since in the present study the amount of RNA in the population more than doubles in the period from day 10 to day 25 (Table 2), it can be inferred that the population must synthesize and accumulate RNA in this time period. Since the RNA profile (Figures 2A and 3A) does not change greatly, it can be assumed that most of this synthesis is rRNA. Superimposed on the decline in RNA synthesis in maturing erythroid cells of *X. laevis* is a decline in the proportion of α -amanitin-insensitive incorporation in nuclei isolated from these cells (Hentschel and Tata, 1978). This suggests that the cells shift from mostly RNA polymerase I-dependent rRNA synthesis to mostly RNA polymerase II-dependent pre-mRNA synthesis. Consistent with this, in late stage and mature cells of the erythropoietic series studied here, the amount of rRNA decreases relative to total RNA (Figures 2A and 3A). The small amount of *in vivo* uridine incorporation observed in mature chicken red cells was also mostly into RNA polymerase II-dependent pre-mRNA (Zentgraf *et al.*, 1975).

B. Histone and globin protein synthesis and mRNA levels

The rate of cell division in the developing erythroid population is reflected by the amount of labelled thymidine incorporated into DNA, and labelled amino acid incorporated into replication-dependent H1 and core histones. Rutledge *et al.* (1984) showed that in *X. laevis* both these parameters dropped to low levels by day 20 of recovery from anaemia. Since replication-dependent histone synthesis is paralleled by the levels of mRNAs which code for these histones, the levels of these messages can also be used as an index of cell division rate. Koster (1987) showed that the amount of H1 and core histone message declines greatly between days 17 and 21 of recovery from anaemia. In the present study H4 mRNA can be used as a similar index, levels of which fall to near control levels between days 20 and 25 of recovery (Figure 4). For these experiments, heterologous H4 probes were used in Northern blot analyses, which showed weak signals and high backgrounds (*cf.* the Northern analysis of H4 mRNA in Koster, 1987). The poor quality of these results is due to the probes used, underlining the benefit of using homologous probes where possible. As shown in Appendix 2, the coding region of the probe sequence of sea urchin H4 was 77-78% similar, and that from chicken H4 was 81-85% similar, to three H4 sequences from *X. laevis*.

Rutledge *et al.* (1984) showed that H5 synthesis during erythropoiesis in *X. laevis* continued after the synthesis of core and H1 histones had become undetectable, to at least day 26 of recovery

from anaemia. Peak expression of the protein was in the earliest cells studied, from day 10, after which H5 expression gradually declined. However, in the present study, H5 mRNA levels were shown to rise in the recovery period from day 10 to days 20-25. This inverse relationship between H5 mRNA levels and protein synthesis probably doesn't represent a specific translational control, because overall protein synthesis decreases in this time period as well (Rutledge *et al.*, 1984). This inverse relationship suggests that the developing erythroid cell increases the H5 mRNA level to maintain H5 synthesis in cells with decreasing protein synthesis capability.

H5 mRNA persists as a relatively large proportion of total RNA even in mature red cells (Table 3). However, since the absolute amount of total RNA falls in these cells (Figure 1), the amount of H5 mRNA per cell decreases to low, but significant, amounts (Figure 4). Thus the kinetics of accumulation of H5 mRNA in erythroid cells of *X. laevis* differs from that in chicken in two ways (Affolter *et al.*, 1987). First, peak mRNA levels are highest in chicken erythroblasts, after which these levels steadily decline, and second, H5 mRNA is not detectable in mature chicken erythrocytes.

It has not been shown whether this message remaining in mature *X. laevis* red cells directs protein synthesis *in vivo*. This question was not addressed by Rutledge *et al.*, 1984. Using ³⁵S-methionine labelling, Winning and Browder (1988) showed the synthesis of many proteins in mature *X. laevis* red cells, one of which has the molecular weight expected for H5. Thus the question of whether H5 is made in mature cells remains unresolved. To show

that H5 is synthesized in mature erythrocytes, the cells should be incubated with ^{35}S -methionine, perhaps extracted with 5% perchloric acid (in which only lysine-rich histone and high mobility group proteins are soluble), and the labelled protein precipitated with anti-H5 antibody. This would be followed by electrophoresis and fluorography, comparing the electrophoretic mobility of the precipitated product of *in vivo* labelling with authentic H5.

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As with H5 mRNA, the peak amounts of the globin message, expressed either as a fraction of total RNA or on a per cell basis, are found in day 20-25 cells, with lower amounts of this mRNA persisting in late stage and mature cells (Table 3, Figure 6). It is not known when peak synthesis of globin occurs, but since it is the major protein synthesized, this point should be at the peak of overall protein synthesis, early in recovery (Rutledge *et al.*, 1984). If this is true, then an inverse relationship exists between globin synthesis and α -globin mRNA amounts for the time period from days 10 to days 20-25, similar to that of H5, above.

C. Characterization of the *X. laevis* H5 mRNA

Avian H5 and mammalian H1⁺ mRNAs are polyadenylated (Molgaard *et al.*, 1980; Kress *et al.*, 1986; Alonso *et al.*, 1988). Whether *X. laevis* H5 mRNA is polyadenylated has been addressed in two previous studies. Koster (1987) found that H5 mRNA, as measured by *in vitro* translation, was enriched in the poly(A)⁺ fractions after chromatography on either oligo(dT)-cellulose or poly(U)-sepharose, while globin mRNA was found mainly in the poly(A)⁺ fractions. However, fractionation of day 8 total RNA by oligo(dT)-cellulose chromatography followed by Northern blot analysis tentatively placed the H5 mRNA in the poly(A)⁺ fraction (Rutledge, 1987). In addition, several of the cDNA clones obtained by Rutledge *et al.* (1988) end in poly(A) sequences of up to 15 nt. In the present study, fractionation of day 25 total RNA by oligo(dT)-

cellulose chromatography followed by Northern blot analysis was repeated, showing that H5 and globin mRNAs were both enriched in the poly(A)⁺ fraction in a quantitatively similar manner, and by this criterion it is likely that the H5 message is indeed polyadenylated. This would best be confirmed by examination of the gene sequence (which is as yet uncloned) at the polyadenylation site. Northern blot analysis (Figures 2B and 3B) show that the H5 messages from the various erythroid cell types are all of the same length; thus there is no evidence for progressive shortening of the poly(A) tail of the molecules as the cells mature.

Confirmation of the size of the H5 mRNA used denaturing glyoxal-agarose gel electrophoresis in addition to the more common formaldehyde-agarose gels. Use of standard methods for glyoxal gels (Maniatis *et al.*, 1982; Ogden and Adams, 1987) was frustrated at first by: 1) the requirement for laborious (and unreliable) deionization of commercial glyoxal solutions, 2) the presence of RNase activity in the gels (a problem not found using formaldehyde gels, because the formaldehyde in the gel inhibits RNase), and 3) very low fluorescence of ethidium bromide-stained, glyoxal-treated RNA. These problems were overcome by: 1) preparation of fresh glyoxal solution from solid glyoxal trimeric dihydrate, obviating the need for deionization of the solution (Skopp and Lane, 1988), 2) use of iodoacetate and SDS in the gel to inhibit RNase (Skopp and Lane, 1988), and 3) reversal of the glyoxal-RNA complex before ethidium bromide staining (Villarreal *et al.*, 1979), as described in Materials and Methods (section II.C.3). Since glyoxal is less toxic

than formaldehyde, use of this gel system could provide a less hazardous alternative to formaldehyde gels.

Known sizes of H5 and H1* mRNAs are listed in Table 6. The size of the *X. laevis* H5 mRNA was shown in this study to be 2.1-2.2 kb, longer than expected from previous studies. The length of the *X. laevis* H5 mRNA is twice as long as that of avian H5, and is similar in size to H1* mRNA. The 5' end of the message, missing from the cDNA clones of Rutledge *et al.* (1988), was mapped relative to the coding region by primer extension analysis. The primer used was exactly complementary to a sequence in the coding region of the H5A cDNA, but contained a single mis-match to the H5B cDNA sequence (Appendix 3). For this reason, and because the amount of H5B mRNA in *X. laevis* red cells is thought to be low relative to H5A mRNA (Rutledge *et al.*, 1988), it is expected that the contribution of H5B mRNA to this primer extension analysis was small. Primer extension analysis showed that the *X. laevis* mRNA has an unusually long, 1.25 kb 5' UTR. Thus, *X. laevis* H5 mRNA differs from H1* mRNA in that this H5 message has a long 5' UTR, whereas H1* messages have long 3' UTRs. These sequences may be important in the regulation of translation or mRNA stability, by the action of specific factors that interact with mRNA sequences (Brawerman, 1987).

In primer extension analysis of H5 mRNA, the most intense band was found at only 0.55 kb from the primer (Figure 7, lanes c and d). This indicates a 'strong stop' for reverse transcriptase at 1.2 kb from the putative polyadenylation site, which possibly explains why the longest cDNA obtained by Rutledge *et al.* (1988)

Table 6. Properties of H5 and H1^o mRNAs.

Origin	reference	lengths (kb)				AAUAAA present
		total length ^a	5' UTR ^b	coding ^c	3' UTR ^d	
Chicken	e	0.900 ± 0.05		0.567	0.198	no (uauaaa?)
	f, g	1.05-1.10	0.117, 0.120			
Duck	h	1.05	0.152	0.579	0.213	no
Human	i	2.3	0.306	0.579	1.310	yes
Mouse	j, k	2.1	0.269	0.579	1.1	?
<i>X. laevis</i>	l	1.3-1.5	>0.390	0.585	0.202	no
	m	0.8-1.2 (12s)				
	n	2.1-2.2	1.25			

^a Total lengths were determined by Northern blot analysis, except for *X. laevis* reference m, which employed sucrose gradient centrifugation.

^b including the ATG translation start

^c excluding the translation start (ATG) and stop (TGA, TAA) codons

^d including the translation stop codon, but excluding the poly(A) tail

References:	e	Krieg <i>et al.</i> (1982)	j	Alonso <i>et al.</i> (1988)
	f	Affolter and Ruiz-Carillo (1986)	k	Breuer <i>et al.</i> (1989)
	g	Ruiz-Carrillo <i>et al.</i> (1983)	l	Rutledge (1987)
	h	Doenecke and Tonjes (1984)	m	Koster (1987)
	i	Kress <i>et al.</i> (1986)	n	this thesis

was 1.2 kb. Pretreatment of RNA substrate for reverse transcriptase with methylmercuric hydroxide is effective in increasing the proportion of long cDNAs relative to untreated RNA (Figure 7, lane d *cf.* lane c).

Confirmation of the size of the 5' UTR (and indeed of the 3' UTR) with higher resolution would best be done by mapping of the 5' and 3' ends of the message against genomic clones spanning these points. The cloned H5 gene would be essential for this purpose, and useful for other purposes, such as an examination of the remainder of the 5' UTR and the promotor region. The range of lengths of the poly(A) tails might be measurable by nuclease protection mapping against long poly(dT) molecules.

Most, but not all, poly(A)⁺ messages are polyadenylated 10-30 nt 3' to a putative polyadenylation signal, with a consensus sequence AAUAAA (Birnstiel *et al.*, 1985). However, human H1⁺ is the only known member of this class of histone to have a message with a consensus polyadenylation signal: avian and *X. laevis* H5 mRNAs do not have this sequence.

D. Transcription and stability of *X. laevis* H5 mRNA

In vitro 'run-on' transcription in isolated nuclei allowed detection of small amounts of H5 transcription only in early stages of recovery. Thus transcription of H5 in *X. laevis* nuclei differs from that in chicken, where approximately equal amounts of transcription of H5 were found in permeabilized cells from early polychromatic

erythrocytes and mature red cells (although H5 mRNA is not detectable in mature chicken erythrocytes: Affolter *et al.*, 1987). *In vivo* transcription probably is reflected imperfectly by assays which measure transcription in isolated nuclei or in permeabilized cells, and certainly these assays do not show formation of processed mRNA. *In vivo* transcription would be better measured by *in vivo* pulse-labelling assays using labelled uridine (Ganguly and Skoultchi, 1985). One must consider the effects of endogenous uridine pools in interpreting results in this kind of experiment. Also, since ^3H is the usual label (phosphorylated nucleotides do not cross the cell membrane), this method suffers from low sensitivity. However in the present study, since the amount of H5 mRNA per cell increases in the period from day 10 to day 20-25 (Figure 4), as does the number of cells (Table 2), it can be inferred that H5 mRNA is synthesized and accumulates in this time period.

Since H5 transcription cannot be detected in nuclei from late stage or mature cells, yet H5 mRNA remains present even in mature cells, these data suggest that the H5 message is very stable. A more direct method of assessing the stability of mRNAs involves the use of transcription inhibitors such as actinomycin D or α -amanitin, followed by measurement of the rate of disappearance of specific mRNAs. However, these inhibitors may have unwanted side-effects on the cells. Probably the most direct way to assay mRNA stability is by pulse-labelling cells *in vivo* with labelled uridine, and then noting the disappearance of specific labelled message during a 'chase' period (taking into account endogenous uridine pools and low sensitivity,

mentioned above). A promising new method for monitoring mRNA decay in a cell-free system has been developed (Ross and Kobs, 1986).

E. Globin transcription

Primary transcripts of globin genes have been the subject of numerous studies. In the rabbit, the only primary transcript was a 15s pre-mRNA, and no flanking intergenic sequences were detected (Grosveld *et al.*, 1981). Most globin pre-mRNA in the duck is 1.6 kb, but globin-containing sequences of up to 15 kb have been detected (Reynaud *et al.*, 1980). Analysis of RNA species which contained globin gene sequences in the mouse and in MEL cells showed, in addition to the 0.75 kb mature mRNA, a rapidly-labelled poly(A)⁺ species of about 1.5 kb, and a rapidly-labelled poly(A)⁻ species of about 5 kb (Bastos and Aviv, 1977). Later studies of MEL cells using 'run-on' transcription in isolated nuclei showed that most primary transcripts begin at the cap site, and that most of the transcripts terminate at a site 1.4 kb 3' to the polyadenylation site, making a primary transcript with a total length of about 2.8 kb (Hofer and Darnell, 1981; Hofer *et al.*, 1982). A more recent study of β -globin transcripts in MEL cells showed precursor species of 1.8-1.9 kb, 3.7 kb, and >4.5 kb in length (Winicov, 1988). These precursor molecules were initiated in the promotor region of the gene, lacked a poly(A) tail, were processed slowly, and contained intron and

flanking sequences not found in mature mRNA (Weidner and Winicov, 1989).

In the present study, putative precursors of α -globin mRNA with lengths of 2.8 kb and >6 kb were found in substantial amounts in all stages except mature red cells (Figure 2A). The results of the present study are surprising because one would not expect that pre-mRNA molecules, considered short-lived, would be present in amounts large enough to show as strong bands in Northern blots of total RNA (Birnstiel *et al.*, 1985). That the putative precursor molecules are present in substantial levels in recovering cells, but not in mature cells, implies that the molecules are slowly processed in *X. laevis* erythroid cells, as suggested for those in MEL cells (Weidner and Winicov, 1989). However, two experiments described in section III.D indicate that these bands may be artefactual. In the first experiment, employing oligo(dT)-cellulose chromatographic fractionation of RNA, the putative precursor molecules were found in substantial amounts in neither the poly(A)⁺ nor the poly(A)⁻ fractions, when it was expected that the putative precursors, being unprocessed and unadenylated, would be found in the poly(A)⁻ fraction. In the second experiment, in a sample of poly(A)⁺ RNA which was incubated in primer hybridization solution, the amount of the >6 kb band greatly increased relative to that found in control poly(A)⁺ RNA. Since rRNAs, which contain much secondary structure, had electrophoretic mobilities which accurately reflected their lengths in the formaldehyde gels used (Table 4), the possibility that the putative α -globin mRNA precursors are electrophoretic artefacts

due to incomplete denaturation can likely be eliminated. Thus the nature of the putative α -globin pre-mRNA molecules in *X. laevis* erythroid cells remains unexplained.

In the 'run-on' transcription assay, globin transcription was detected at all stages of recovery, but it was not clear whether mature nuclei transcribed this gene (Figure 9). Winning and Browder (1988) showed transcription of the β -globin gene in *X. laevis* nuclei from all erythroid stages from erythroblasts to mature erythrocytes, albeit small amounts in the latter nuclei. This is similar to a previous study which showed that globin transcription in mature *X. laevis* red cell nuclei was very low, similar to the (presumably artefactual) transcription of globin in liver nuclei (Tata and Baker, 1985). Thus if transcription of globin occurs in mature *X. laevis* red cell nuclei, it is near the limit of detection. Transcription of β^A -globin was found in large amounts in permeabilized chicken erythroid cells from all stages from erythroblasts to mature erythrocytes, but its mRNA was not detectable in mature chicken red cells (Affolter *et al.*, 1987). In proliferating erythroid cells of the chicken (erythroblasts and polychromatic erythrocytes), transcription of β^A -globin is much less than that of histone H2A, yet the amount of β^A -globin mRNA is greater than 20-fold more than the amount of H2A mRNA in these cells, emphasizing the extreme stability of this globin mRNA (Affolter *et al.*, 1987).

F. Perspectives

The erythropoietic system used in the present study, based on recovery from anaemia in *X. laevis*, has been shown in the present thesis and in several other studies to be useful as an example of erythropoiesis, as a model of terminal differentiation, and as a model system for the study of gene expression. This system allows easy access to erythroid cells in large amounts, and has an advantage over erythropoietic systems in cell culture in that the *X. laevis* cells are not transformed. Fairly homogeneous populations can be obtained (Table 1), but if greater homogeneity is required, then the populations can be fractionated further, for example by density gradient centrifugation (Hentschel and Tata, 1978; Adams and Neelin, 1972). Alternatively, without further fractionation, the contributions of minority cell types to the metabolic processes studied could be computed and simply subtracted from the total (Winning and Browder, 1988).

Several themes concerning the expression and role of H5 were developed in the Introduction to this thesis, which suggest directions for further study. For example, the state of phosphorylation of avian H5 is important in erythropoiesis (section I.E.2.a.), but the role of phosphorylation of other H5s or of H1* has not been addressed. It would be of interest to determine if phosphorylation of H5 or H1* is important in non-avian systems. A recurring theme in this thesis is the recognition that an important component in the regulation of gene expression is the regulation of mRNA stability. Different

mRNAs have different stabilities, and this is best explained by the interaction of specific factors with specific mRNA sequences (Brawerman, 1987). Thus it can be asked: What is the mechanism, or what are the factors and sequence elements, which stabilize globin and (likely) H5 mRNAs in erythroid cells when other mRNAs are degraded? Mechanisms and factors controlling mRNA stability in other contexts are just beginning to be recognized (Shaw and Kamen, 1986; Ross *et al.*, 1987; Brewer and Ross, 1989; Bernstein and Ross, 1989).

The function of H5 in erythropoiesis was not addressed in this study. This would best be done by making the cellular content of H5 an experimental variable, and such manipulation can probably be done only in cell culture erythropoietic systems. For example, a transfected H5 gene could be expressed in MEL or AEV cells (with and without induced differentiation) to determine its effect on proliferation and differentiation. Results of the present study, in which it is inferred that the H5 message is stable in *X. laevis* red cells, suggest that an H5 transgene message would also be stable in other erythroid cells. Another method by which H5 expression could be manipulated involves 'reverse genetics', wherein the gene is inactivated by recombination with introduced mutant sequences homologous to the target gene (Thomas and Capecchi, 1986). Avian H5 and murine H1* would be appropriate target genes for this technique because there is only one of each gene per haploid genome (Ruiz-Carrillo *et al.*, 1983; Alonzo *et al.*, 1988). Another method by which the amount of endogenous H5 or H1* expression could be

decreased is by induced expression of transgenic anti-sense sequences complementary to the H5 or H1* mRNAs (Weintraub, 1990). Using either of these techniques, one could determine the effect of the absence of H5 in AEV cells, or the absence of H1* in MEL cells, on proliferation, differentiation, and chromatin structure.

V. APPENDICES

Appendix 1. Probe confirmations

a. XLH5A-1

This clone is a 313 b partial cDNA of *X. laevis* H5 in M13mp9 (Rutledge *et al.*, 1988). The clone actually used in this study was XLH5A-1X, a reversal of the insert of this clone in M13mp8. Double digestion of its RF DNA with *Hind*III and *Eco*RI releases a 0.34 kbp fragment from the 7.25 kbp vector (Figure 10 A, lane d). Digestion with *Hind*III linearizes the RF to give a fragment slightly larger than the vector alone (lane c, 7.59 kbp *cf.* lane d, 7.25 kbp). Both XLH5A-1 and -1X were partially sequenced, agreeing with the reported sequence.

b. pXG6C1

This is a 0.64 kb α -globin cDNA inserted in the *Pst*I site of pBR322 (Kay *et al.*, 1980, 1983). Digestion of the recombinant plasmid with *Pst*I releases the insert from the vector (Figure 10 C, lane g). Digestion of the plasmid with *Hae*III (Figure 10 C, lanes d and e) results in a complex 'fingerprint' of fragments which matches exactly that shown for an identical digest in Figure 1 in Kay *et al.*, (1980).

Figure 10. XLH5A-1(X) and pXG6C1 digests and maps

Panel A shows digests of XLH5A-1X. Lane a, marker phage λ digested with *Hind*III. Lane b, marker pBR322 digested with *Hinf*I. Lane c, XLH5A-1X digested with *Hind*III. Lane d, XLH5A-1X digested with *Hind*III and *Eco*RI. Panel B shows a partial map of both XLH5A-1 and XLH5A-1X.

Panel C shows restriction analyses of pXG6C1. Lanes a and h, marker phage λ digested with *Hind*III. Lanes b and f, marker pBR322 digested with *Alu*I. Lane c, marker pBR322 digested with *Hae*III. Lanes d and e, two identical clones of pXG6C1 digested with *Hae*III. Lane g, pXG6C1 digested with *Pst*I. Panel D is a map of pXG6C1. The insert is [*Pst*I, poly(dG) (about 25 bp), partial 5' UTR (21 bp), ATG + coding region + TAA (429 bp), 3' UTR (110 bp), poly(A) (30 bp), poly(dC) (about 25 bp), *Pst*II], in the orientation shown in the vector pBR322.

A **a b c d****B** **C** **a b c d e f g h****D**

c. pGC42

This is a 1.1 kbp *HhaI* fragment spanning the sea urchin *Strongylocentrotus purpuratus* H4 gene, flanked by *HindIII* linkers, and cloned into the *HindIII* site of pBR322 (Childs *et al.*, 1979). Digestion with *HindIII* releases the insert (here measured as 0.89 kbp, Figure 11 A, lane c) from the vector. This is in approximate agreement with Weinberg *et al.* (1977) who measured the H4 *HhaI* fragment as 0.94 kbp. Double digestion of vector alone with *HindII* and *HindIII* gave fragments measured at about 3200, 625, and 540 bp (Figure 11 A, lane b), when sizes of 3256, 623 and 483 were expected. Double digestion of the recombinant with these enzymes (lane d) gave fragments measured as about 3200, 635, and 490 bp, sizes expected of the vector-derived fragments. This digestion also produced a 300 bp fragment, which probably is the fragment [*HindIII* (linker) *HhaI* - 292 bp - *HindII* (in the codon for Arg-35)] from the insert (Grunstein *et al.*, 1981). Three other bands from this double digest are evident, with sizes measured as 2.65, 0.99, and 0.86 kbp, the last of which may be the remainder of the insert.

d. pCH3dR8

This is a 1.21 kbp fragment spanning the chicken histone H4 gene, cloned into the *EcoRI* site of pBR322 (Sugarman *et al.*, 1983). Digestion of the plasmid with *EcoRI* releases the insert from the vector (Figure 11 B, lane b). To confirm the orientation, the plasmid

**Figure 11. pGC42 digest, and
pCH3dR8 digest and map**

Panel A shows restriction analysis of pGC42. Lane a, marker pBR322 digested with *Hinf*I. Lane b, marker pBR322 digested with *Hind*II and *Hind*III. Lane c, pGC42 digested with *Hind*III. Lane d, pGC42 digested with *Hind*II and *Hind*III. Lane e, marker phage λ digested with *Hind*III.

Panel B shows restriction analysis of pCH3dR8. Lanes a and d, marker phage λ digested with *Hind*III (note that the 4.4 kb band is faint). Lane b, pCH3dR8 digested with *Eco*RI. Lane c, pCH3dR8 digested with *Ava*I. Panel C shows a map of pCH3dR8.

was digested with *Ava*I, which cuts once in the insert and once in the vector. This digestion gave fragments measured as 3.24 and 2.33 kbp (lane c), consistent with the orientation shown (Figure 11 C).

e. MV2

MV2 was made as described in section 11.1. When MV2 was linearized with *Eco*RI, a single fragment results (expected size 4027 bp, Figure 12, lane d). The 862 bp *Eco*RI-*Hind*III fragment is excised from the vector pUC119 on double digestion with these two enzymes (lane e). Orientation of the insert was confirmed using *Pvu*II and *Sca*I, both of which cut in both the insert and the vector. Expected sizes for *Pvu*II digestion are 2893, 986, and 148 bp, and those for *Sca*I are 2479 and 1548 bp (lanes f and g). Also, the region near the *Eco*RI site was sequenced, confirming that the molecular manipulations did not produce artefacts in that region of the clone.

Figure 12. MV2 digest and map

Panel A shows restriction analysis of MV2. Lane a, marker phage λ digested with *Hind*III. Lane b, marker pBR322 digested with *Hinf*I. Lane c, MV2, uncut. Lane d, MV2 digested with *Eco*RI. Lane e, MV2 digested with *Eco*RI and *Hind*III. Lane f, MV2 digested with *Pvu*II. Lane g, MV2 digested with *Sca*I. Panel B shows a map of MV2.

a b c d e f g

Appendix 2

Figure 13. Comparison of histone H4 coding sequences

Sequences for the sea urchin *Strongylocentrotus purpuratus*, (Sp, Grunstein *et al.*, 1981), three from *Xenopus laevis* (Xl, Perry *et al.*, 1985), and from chicken (Ch, Sugarman *et al.*, 1983) are presented, taken from Wells and McBride (1989). These sequences code for identical proteins, except that SpH4 aa 73 is cysteine, whereas it is threonine in the other four.

Amino acid (Sp)		Ser	Gly	Arg	Gly	Lys	Gly	Gly	Lys
Sp	ATG	TCA	GGT	CGA	GGA	AAA	GGA	GGA	AAG
Xl-A	---	--T	--A	--T	--C	---	---	---	---
Xl-B	---	--T	--A	--C	--C	---	---	---	---
Xl-C	---	--T	--A	A-A	--C	--G	--C	---	---
Ch	---	--T	--C	A-A	--C	--G	--C	--G	---

		Gly	Leu	Gly	Lys	Gly	Gly	Ala	Lys	Arg	His	Arg
Sp	9	GGA	CTC	GGA	AAG	GGT	GGT	GCC	AAA	CGT	CAT	CGC
Xl-A		--C	T-A	--G	--A	--A	---	---	--G	--G	--C	A-G
Xl-B		--C	T-A	--G	--A	--A	--C	--T	--G	--G	--C	A-G
Xl-C		--T	--G	--C	--A	--A	--C	---	---	---	--C	A-G
Ch		--G	---	--C	--A	--G	---	---	--G	--C	--C	---

		Lys	Val	Leu	Arg	Asp	Asn	Ile	Gln	Gly	Ile	Thr
Sp	20	AAG	GTT	CTA	CGA	GAT	AAC	ATC	CAA	GGC	ATC	ACC
Xl-A		---	--G	--G	--G	---	---	--T	--G	--T	---	--T
Xl-B		---	--G	T-G	--G	---	---	---	--G	--T	---	--T
Xl-C		---	--G	--G	--G	---	---	---	--G	---	---	---
Ch		---	--G	--G	--C	--C	--T	---	--G	---	---	---

		Lys	Pro	Ala	Ile	Arg	Arg	Leu	Ala	Arg	Arg	Gly
Sp	31	AAG	CCT	GCA	ATC	CGT	CGA	CTN	GCT	AGA	AGG	GGA
Xl-A		--A	--C	--C	---	--C	--C	--G	--C	C-G	--A	--G
Xl-B		--A	--C	--C	---	--C	---	--G	--C	C-G	--A	--G
Xl-C		---	--C	--C	---	--C	--C	--C	--A	C-C	--A	--G
Ch		---	--G	--C	--T	--C	--C	--G	--G	C-G	C-C	--C

		Gly	Val	Lys	Arg	Ile	Ser	Gly	Leu	Ile	Tyr	Glu
Sp	42	GGT	GTC	AAG	AGG	ATC	TCT	GGT	CTC	ATC	TAC	GAA
Xl-A		--A	---	---	--C	---	--A	---	---	---	---	--G
Xl-B		--A	---	---	--C	---	--A	---	---	---	---	--G
Xl-C		--A	---	---	C-C	---	--C	--C	---	---	---	--G
Ch		--C	---	---	C-C	---	--G	--G	---	---	---	---

	53										
	Glu Thr Arg Gly Val Leu Lys Val Phe Leu Glu										
Sp	GAG ACA CGC GGT GTA CTG AAG GTC TTC CTG GAG										
X1-A	--- --T --T --C --T --C --- --T --- --A ---										
X1-B	--- --T --T --C --T --T --- --T --- --A ---										
X1-C	--- --T --- --G --G --C --A --T --- --- ---										
Ch	--- --G --- --C --G --C --- --- --- --T ---										
	64										
	Asn Val Ile Arg Asp Ala Val Thr Tyr Cys Glu										
Sp	AAT GTC ATC CGT GAT GCA GTC ACC TAC TGC GAG										
X1-A	--- --T --T --A --C --C --- --- --- ACA --A										
X1-B	--- --T --T --A --C --T --- --- --- ACA --A										
X1-C	--C --T --- --G --C --C --- --- --- AC- ---										
Ch	--C --- --- --C --C --C --- --- --- AC- ---										
	75										
	His Ala Lys Arg Lys Thr Val Thr Ala Met Asp										
Sp	CAC GCT AAG CGA AAG ACT GTC ACA GCC ATG GAC										
X1-A	--- --C --- A-G --- --A --T --C --- --- --T										
X1-B	--- --- --- A-G --- --- --T --C --- --- --T										
X1-C	--- --C --- A-G --- --C --T --C --T --- --T										
Ch	--- --C --- A-G --- --G --- --G --- --- ---										
	86										
	Val Val Tyr Ala Leu Lys Arg Gln Gly Arg Thr										
Sp	GTG GTG TAT GCA CTA AAG AGG CAG GGT CGT ACA										
X1-A	--- --- --C --G --C --- C-C --- --C --C --T										
X1-B	--- --- --C --T --G --- C-C --- --C --C --T										
X1-C	--- --- --- --T --G --- C-C --A --A --C --T										
Ch	--- --C --C --G --C --- C-C --- --A --C --C										
	97										
	Leu Tyr Gly Phe Gly Gly										
Sp	TTG TAC GGC TTC GGC GGC TAA										
X1-A	C-C --- --- --- --A --- ---							vs. Sp	%	vs. Ch	%
X1-B	C-C --- --- --- --A --- ---							235	77	256	84
X1-C	C-- --- --A --- --- --T --- ---							236	77	248	81
Ch	C-C --- --- --- --A --T ---							238	78	261	85

^a The number of sequence matches over the coding region of 306 nucleotides, excluding the translation start and stop codons

Appendix 3.

Figure 14. Sequences of *X. laevis* H5 cDNAs

Reproduced from Rutledge *et al.* (1988). Shown are the nucleotide sequences of the *X. laevis* H5A (upper) and H5B (lower) cDNAs. The A-rich region, in which 15 of 18 nt are A, corresponds to nt 450-467. The primer 5-D24R used in section II.H and III.E (primer extension analysis of the H5 mRNA) is a 29-mer oligonucleotide (5'-CAA-TAT-CAT-GTC-AGA-GTA-CTT-AGG-GTG-GT-3') which was made to be complementary to the region from leu-33 to asp-24.

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