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CHANGES IN THE HEAT SHOCK RESPONSE AND HSP70 AND CONSTITUTIVE GENE TRANSCRIPTION DURING ERYTHROPOIESIS OF XENOPUS LAEVIS

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled CHANGES IN THE HEAT SHOCK RESPONSE AND HSP70 AND CONSTITUTIVE GENE TRANSCRIPTION DURING ERYTHROPOIESIS OF XENOPUS LAEVIS, submitted by ROBERT S. WINNING in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

As erythroid cells of *Xenopus laevis* differentiate, their capacity for transcription and protein synthesis become progressively restricted. Mature erythrocytes are thought to be transcriptionally inert and have very low levels of protein synthesis. In this study, actual and potential synthetic activity have been examined by comparing the patterns of protein synthesis under ambient and heat shock conditions and measuring transcriptional activity of selected genes during erythropoiesis. As erythropoiesis proceeds, there is a progressive decrease in the number of heat shock proteins (hsps) whose synthesis is induced at elevated temperatures. In spite of this repression, mature erythrocytes respond to heat shock with the induction of synthesis of one protein, hsp70. In addition, an hsp70-like protein is synthesized constitutively in orthochromatic erythroblasts, the final erythroid cell stage before maturation into erythrocytes. An *in vitro* nuclear runoff transcription assay was used to study transcription of the hsp70 gene and several other genes. Globin and actin gene transcription decline during erythropoiesis to negligible levels in erythrocytes. Ribosomal gene transcription , however, remains relatively high in erythrocytes. Hsp70 gene transcription shows both stage-specific and heat shock-dependent transcription. Orthochromatic erythroblasts and erythrocytes have heat shock-dependent hsp70 gene transcription, whereas the gene is transcribed at the earlier erythroblast stages at ambient temperature as well as during heat shock. The constitutive hsp70 transcripts may be stored for utilization in constitutive synthesis of the hsp70-like protein in orthochromatic erythroblasts. Transcription of actin and keratin-related sequences is also induced in erythrocytes by heat shock. These data indicate that although they become synthetically repressed during differentiation, erythrocytes are less inert than had previously been thought.

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INTRODUCTION

One of the fundamental unexplained problems in the study of development of multicellular organisms is that of cell determination. The unicellular zygote produced by fertilization must give rise to a multitude of diverse cell types in the adult. The mechanics of the process of differentiation by which this diversity is acquired and the state of the fully differentiated cell have been the subjects of scientific inquiry for several decades.

The first theory that attempted to explain the mechanism of differentiation was the theory of qualitative nuclear division proposed by Wilhelm Roux in 1883 and elaborated upon by August Weismann in 1892 (Browder, 1984; Leonard, 1980). This theory proposed that the primary determination in development was effected by the nucleus and the chromatin contained within it. Chromatin was assumed to consist of different determinants that would be sifted apart by qualitative cell division until each daughter cell contained only one determinant that would specify the fate of that cell. Cleavage would thus result in a mosaic of self-differentiating cells.

Evidence supporting this theory was obtained by Roux (1888) in an experiment in which he used a hot needle to kill one blastomere of a two-cell *Rana esculenta* blastula. The remaining blastomere developed abnormally, suggesting to Roux that the surviving blastomere lacked some determinants that had been inherited only by the killed blastomere.

This theory, however, was refuted by the results of Driesch (1892), who observed that individual blastomeres of the sea urchin *Echinus microtuberculatus* separated at the two or four cell stage in calcium-free sea water were capable of developing into morphologically normal but undersized larvae. Roux's theory was further disproved by the work of Boveri (1902), who established that normal development is dependent on each cell containing the normal chromosomal complement. Boveri had observed that cleavage in dispermic sea urchin eggs was multipolar, involving three, or more often four, centrosomes. As a result,

abnormal numbers of chromosomes were inherited by most blastomeres. When separated, blastomeres with abnormal chromosomal numbers developed abnormally, and it was observed that different abnormalities were associated with different combinations of chromosomes. Only rarely did blastomeres develop into morphologically normal larvae, and those were found to contain the normal complement of chromosomes.

It has since been demonstrated that every differentiated diploid cell of an organism contains (with few exceptions) the same quantity of DNA (Mirsky and Ris, 1949). In addition, DNA-DNA hybridization studies have shown that DNA samples from mouse embryo, mouse brain, kidney, thymus, spleen, and liver are equal in their ability to compete with labeled mouse L-cell DNA for complementary sequences in mouse embryo DNA (McCarthy and Hoyer, 1964). It was concluded from these experiments that all DNA nucleotide sequences are present in each somatic cell in the same relative proportions.

These and other similar studies have led to the evolution of the theory of variable gene expression, which is the currently accepted view of differentiation. This theory states that the genomes within the differentiated cells of an organism are essentially equivalent and that differentiation depends on selective variation in gene activity (Stedman and Stedman, 1950). Only the genes required to give a cell its particular identity are expressed; the rest of the genome remains repressed.

If such is the case, a question remains as to the nature of the repression, whether it involves permanent alteration of the genetic material or simply a potentially reversible inhibition of transcription of genes whose expression is not required in a given cell type. This question has been addressed by studies involving nuclear transplantation. In these experiments, nuclei from differentiating or differentiated amphibian cells are microinjected into enucleated eggs of the same species. In some cases *Rana pipiens* blastula nuclei (Briggs and King, 1952) and even *Xenopus laevis* tadpole intestinal epithelial cell nuclei (Gurdon,

1962) have been shown to be capable of directing the development of enucleated eggs into normal larvae and even sexually mature adults (Gurdon and Uehlinger, 1966). These studies, apart from providing further evidence for the variable gene activity theory, suggest that the generalized gene repression associated with differentiation is reversible under the proper conditions. The nature of the modifications to the nuclei of differentiated cells, however, remains to be investigated.

Amphibian erythrocytes, which - unlike their mammalian counterparts - retain their nuclei, have been used by a number of researchers in nuclear reactivation studies. Erythrocytes have been used in such studies because they have been thought to represent an extreme example of cell specialization. The *Xenopus* erythrocyte, in particular, has been described as being probably closer to total synthetic shutdown than any other nucleated cell (Maclean *et al.*, 1973). Nuclei from *Rana pipiens* erythrocytes, when microinjected into oocytes of the same species, show an increase in volume, dispersion of chromatin, and an increase in transcription (Leonard *et al.*, 1982). These nuclei have also been shown to be capable of directing development of oocytes, after maturation and activation, into swimming tadpoles (DiBerardino and Hoffner, 1983). Erythrocyte nuclei from *Xenopus laevis*, when transplanted into *Xenopus* oocytes, show reactivation of the 4S, 5S, and 5.8S RNA genes (Wakefield *et al.*, 1983).

The normal synthetic properties of *Xenopus laevis* erythrocytes have been investigated by Maclean and various co-workers. Protein synthesis was studied in *Xenopus* erythrocytes (Maclean *et al.*, 1969) by incubating the cells *in vitro* in ^{3}H-leucine and fractionating protein on a CM-cellulose column. The protein concentration of each fraction was then determined by measuring absorbance at 410nm, and the radioactivity of each fraction was measured by scintillation counting. The only major protein fraction detected was globin, and the majority

of the incorporated ^{3}H-leucine was found in the globin fraction. When autoradiography was performed on smears of fixed labeled erythrocytes, it was determined that no more than 30% of all erythrocytes in an individual were engaged in protein synthesis, although there was considerable variation between individuals. It was therefore concluded from this study that *Xenopus* erythrocytes were not very active in protein synthesis, and the synthesis that was detected was almost exclusively of globin.

RNA synthesis was studied in these cells by examining ^{3}H-uridine incorporation (Maclean *et al.*, 1973). Labeled RNA preparations were run on polyacrylamide gels that were scanned to determine absorbance at 260nm in order to localize major RNA species. Gel lanes were then sliced into 1mm sections, which were scintillation counted to determine the radioactivity incorporated in each section. Total incorporation of ^{3}H-uridine was low relative to that of chicken erythrocytes (Madgwick *etal.*, 1972), and the majority of incorporated radioactivity was found to localize in three major RNA bands, which corresponded to 28S, 18S, and 4S RNAs. Autoradiography of ^{3}H-uridine-labeled erythrocyte smears demonstrated that only 12% of the cells showed any detectable incorporation of ^{3}H-uridine, although a more recent study showed that only 0.2% of the cells incorporated labeled uridine (Thomas and Maclean, 1975). In addition, a previous study had shown that globin synthesis in erythrocytes was resistant to the transcription inhibitor actinomycin D, suggesting that transcription was not required for protein synthesis in mature erythrocytes. It was therefore concluded that the *Xenopus* erythrocyte nucleus is as transcriptionally inert as any known.

A subsequent study of transcription in isolated *Xenopus* erythrocyte nuclei (Hentschel and Tata, 1978) supported this conclusion. RNA polymerase II was determined to be present in erythrocyte nuclei and engaged in transcriptional complexes, but restricted in its ability to elongate RNA chains *in vitro*. The observation by Maclean *et al.* (1973) of ribosomal

transcription in erythrocytes was contradicted by this study, which claimed that RNA polymerase I is absent from these cells.

The experimental procedures used in these studies, however, were not very sensitive, so the conclusions drawn from the results must be viewed with some caution. Also, the analyses were limited to constitutive synthesis and did not address inducible synthesis. Techniques in molecular biology have been refined greatly in the years since the above experiments were performed, which allows for a far more sensitive assay of synthetic activity. In addition, the use of probes for specific proteins and RNAs allows the analysis of the synthesis of specific synthetic products, including the products of inducible genes, but as yet no such investigations have been reported on *Xenopus* erythrocytes.

The amphibian erythroid system also offers an excellent opportunity to study changes in gene expression within a cell lineage as the cells differentiate. Grasso and Shephard (1968) reported that induction of total anemia in newts resulted in the precocious release of erythroid precursor cells into the peripheral circulation. After release, the precursor cells differentiated synchronously so that most of the cells were at the same stage of development at any given point. By obtaining blood samples at various times after the induction of anemia, it was possible to obtain fairly homogeneous populations of each stage of erythroid cell.

Anemia was induced using the hemolytic agent phenylhydrazine. This drug acts as an oxidant upon entry into erythrocytes, causing the conversion of hemoglobin to methemoglobin (Emerson *et al.*, 1941), which contains iron in the ferric rather than the normal ferrous state. Methemoglobin is unable to bind oxygen (Jaffe, 1964), and the reduced oxygen pressure that results in the cell induces new hemoglobin synthesis (Marriott, 1968). This accumulation of methemoglobin and hemoglobin molecules renders the cells osmotically fragile and leads ultimately to lysis (Emerson et al., 1941).

In the newt, hemolysis was found to be complete within 48 hours after a single intraperitoneal injection with phenylhydrazine (Grasso and Shephard, 1968). This degenerative phase was then followed by a period of leukocytic generation from 3-14 days after injection and an erythroid regeneration phase from days 13-32. Immature erythroid cells released into the circulation in response to anemia were not affected by the drug.

Phenylhydrazine has been used to induce anemia in *Xenopus laevis* so that morphological and synthetic changes occurring in differentiating erythroid cells could be examined (Thomas and Maclean, 1974; Thomas and Maclean, 1975). Anemia was induced in this case by two injections of 0.5% phenylhydrazine on two successive days. Complete destruction of mature erythrocytes was shown to occur within 12 days. As in the newt, anemia resulted in a wave of erythropoiesis and the premature release of immature erythroid cells into the circulation. The erythroid cell population was more heterogeneous than in the newt, but it was still possible to delineate a developmental series of erythroid cell stages and determine the synthetic activity of each stage relative to the other stages. The methods used to determine synthetic activity were the same as those described previously (Maclean et al., 1969; Maclean et al., 1973).

The first erythroid precursor cells released into the circulation in response to anemia are basophilic erythroblasts, so named because their cytoplasm stains blue with the basic stain in a Wright's stain. These cells are active in DNA and RNA synthesis but - according to Thomas and Maclean - they are inactive in protein synthesis.

The basophilic erythroblasts differentiate into polychromatophilic erythroblasts. Hemoglobin accumulation is first detected in these cells (as determined by staining with o-dianisidine, an agent that specifically stains hemoglobin), and they are very active mitotically, with DNA synthesis reaching a peak during this stage.

Polychromatophilic erythroblasts then differentiate into orthochromatic erythroblasts,

which begin to show morphological similarities to erythrocytes and show some shrinkage of their nuclei. In spite of this, orthochromatic erythroblasts are reported to be the most active of all erythroid cells in terms of RNA and protein synthesis.

This study by Thomas and Maclean on the synthetic activity of immature *Xenopus* erythroid cells suffers from the same weaknesses as the earlier reports on the synthetic activity of erythrocytes. The techniques used are far less sensitive than those commonly used at present. In addition, only constitutive synthesis was examined, whereas inducible synthesis and the synthesis of specific RNAs and proteins were not.

One system that would provide a more complete characterization of the synthetic capacity of *Xenopus* erythroid cells is the heat shock (hs) system. This system has many benefits for such an investigation. Gene expression is inducible by heat, so that the transcriptional state of the heat shock genes can be easily controlled. Many heat shock gene sequences have also been cloned, which allows the analysis of the synthetic activity of specific genes.

The heat shock response was first reported by Ritossa (1962), who observed the induction of novel puffs in polytene chromosomes from salivary glands of *Drosophila busckii* that had been incubated at 30°C, 5° above the normal environmental temperature. Autoradiography of polytene chromosomes after incorporation of ^{3}H-uridine into RNA synthesized on the chromosomes demonstrated that the puffs induced by heat shock were sites of active RNA synthesis (Ritossa, 1964). Puffs existing at normal temperatures were found to regress during heat shock and were shown to cease synthesis of RNA. When radiolabeled proteins from normal and heat-shocked *Drosophila* salivary gland cells were analyzed by SDS-polyacrylamide gel electrophoresis (Tissieres et al., 1974), it was observed that the synthesis of most proteins was repressed by heat shock, whereas the synthesis of a

small set of proteins, termed heat shock proteins (hsps), was induced. The number of heat-induced proteins approximately equalled the number of heat-induced chromosomal puffs, and it was proposed that the inducible puffs were responsible for the synthesis of mRNA used to synthesize the heat shock proteins.

A heat shock response has since been reported for all organisms in which it has been sought, including yeast (Miller et al., 1979), plants (Barnett et al., 1980), human cells (Slater et al., 1981), bacteria (Yamamori et al., 1978), and *Xenopus* (Bienz, 1982). It also appears to be more of a generalized stress response than it first appeared, as the response can be induced by a large number of non-heat stresses, including oxidizing agents, certain metals, amino acid analogs, steroid hormones, glucose deprivation, and viral infection (reviewed in Nover *et al.*, 1984).

Although there is variety in the properties of the heat shock response between organisms, a few common features have become evident. The dominant hsps in all organisms are mostly acidic proteins and fall into one of three size classes: large hsps with relative molecular weights (M_r) of 80-100 kD, hsps with M_r of 68-72 kD (referred to collectively as hsp70), and small hsps with M_r of 15-30 kD. In most organisms hsp70 is the major hsp in terms of level of synthesis, and there is a high degree of conservation of this protein among species. Antibody to chicken hsp70 cross-reacts with hsp70 from *Drosophila*, *Xenopus*, mouse, human, and yeast (Kelley and Schlesinger, 1982). In addition, gene sequences from *Drosophila* show 85% homology to the mouse hsp70 gene (Moran et al., 1983), 74% homology to the *Xenopus* hsp70 gene (Bienz, 1984), 64-72% homology to yeast hsp70-like genes (Ingolia, et al., 1982), and 48% homology to the *E. coli dnaK* protein gene (Bardwell and Craig, 1984).

In *Drosophila*, the hsp70 gene has been found to consist of a family of five related

genes, which are 96% homologous to each other according to nucleotide sequence (Ingolia et al., 1980; Karch et al., 1981). In *Xenopus*, hsp70 has also been shown to be encoded by a family of genes (Bienz, 1984). Four different genes have been identified by restriction mapping, but so far only one has been sequenced.

Another family of genes related in sequence to the hsp70 genes has been identified in *Drosophila* (Ingolia and Craig, 1982). These genes, called heat shock cognate genes, are expressed at normal temperatures but not during heat shock. The four cognate genes are 75% homologous to the heat-inducible hsp70 gene family and show 76% homology among themselves (Craig et al., 1983). Heat shock cognate proteins have also been identified in yeast (Ingolia et al., 1982) and in mouse (Hughes and August, 1982).

Early work by Ritossa (1964), as outlined previously, demonstrated that heat shock causes induction of RNA synthesis at several specific sites, and cessation of transcription at other sites. This suggested that the heat shock response is regulated, if not otherwise, at least at the transcriptional level. This evidence was supported by *in situ* hybridization studies, in which mRNA was labeled with ^{3}H-uridine in cultured *Drosophila* cells during heat shock, then hybridized to polytene chromosomes (Spradling et al., 1975). The labeled heat shock mRNAs hybridized to seven sites that did not bind mRNA from control cells. The seven sites corresponded almost exactly to the regions of puffing observed by Ritossa. Since then, the cloning of many heat shock genes has been accomplished, allowing the use of these sequences as radiolabeled probes. Such probes have been hybridized to RNA that has been electrophoretically separated on denaturing agarose gels and transferred to nitrocellulose (Northern blotting), confirming the heat-induced accumulation of heat shock mRNAs in many organisms (for a review, see Nover et al., 1984). One exception to this seems to be the *Xenopus* oocyte. Whereas the heat shock response in *Xenopus* somatic cells is regulated at

the transcriptional level (Bienz, 1982; Heikkila et al., 1985), the oocyte response does not require *de novo* transcription of the hsp70 gene (Bienz and Gurdon, 1982). Enucleated oocytes and oocytes treated with actinomycin D to inhibit transcription show heat-inducible hsp70 synthesis at approximately the same levels as normal oocytes.

How the transcriptional response is induced is not known, but accumulating experimental evidence is suggestive of a potential mechanism. Analysis of transcription of *Drosophila* hsp70 genes containing deletions in the 5' regulatory region revealed a region upstream from the TATA box that is necessary for heat-inducible promotion (Pelham, 1982). A 15 nucleotide sequence within this region was found to be common to all *Drosophila* heat shock genes. It has since been shown that this consensus sequence is not sufficient for transcriptional activation during heat shock; a second consensus-like sequence upstream from the first is also required (Amin et al., 1985). Digestion studies using exonuclease III (Exo III) revealed two regions of chromatin at the 5' end of the *Drosophila* hsp70 gene that were resistant to digestion with the enzyme during heat shock (Wu, 1984). This suggested that proteins were bound to these regions, but the proteins in question were not identified. Two protein factors were subsequently identified when nuclear extracts from heat-shocked *Drosophila* cells were fractionated chromatographically (Parker and Topol, 1984). One fraction was shown to contain a factor (designated HSTF for heat shock transcription factor) that was required for *in vitro* transcription of the hsp70 gene but not the actin, histone H3, or histone H4 genes. Transcription of the hsp70 gene also requires a general *Drosophila* transcription factor, factor A. Binding of HSTF to the hsp70 gene conferred DNAase I resistance on a region of chromatin upstream from the TATA box; this region contains the heat shock consensus sequence. HSTF was found to be present in both control and heat-shocked cells but was much more active in heat shocked cells, suggesting that it had been modified in response to the stress. The nature of this modification is as yet unclear,

although the protein ubiquitin has been implicated.

Ubiquitin is a small protein (76 amino acids) that is very highly conserved among eukaryotes and occurs in cells either as a free peptide or covalently linked via its carboxy terminus to N-terminal and lysine amino groups of other proteins. It has been postulated that the attachment of ubiquitin to abnormal proteins acts as a signal for the degradation of those proteins by proteinases (Hershko and Ciechanover, 1982). Ubiquitin is also covalently attached to lysine residues of histone H2A (Goldknopf and Busch, 1977; Hunt and Dayhoff, 1977), but this protein is not rapidly degraded. The attachment of H2A to ubiquitin is in rapid equilibrium with the removal of the latter by the enzyme isopeptidase (Seale, 1981).

When cells are heat-shocked, abnormal proteins are no longer degraded efficiently (Munro and Pelham, 1984), presumably because the degradation system, including the ubiquitin attachment system, is overwhelmed by higher levels of denatured proteins. Heat-shocked *Drosophila* cells have greatly reduced amounts of ubiquitinated histones (Glover, 1982), suggesting that isopeptidase remains active but free ubiquitin is no longer available to replace those molecules that are removed.

Accumulation of abnormal proteins has been correlated with the induction of the heat shock response in a temperature-sensitive mouse cell line, ts85 (Finley *et al.*, 1984). These cells have a thermolabile ubiquitin-activating enzyme, so that ubiquitin coupling and protein degradation cease at the non-permissive temperature (39^{o}C). Heat shock proteins are also synthesized at this temperature, even though it is well below the normal heat shock threshold. It has been speculated then, that HSTF could exist in normal cells in an inactive ubiquitinated form, with equilibrium between the attachment of ubiquitin to HSTF and the removal of ubiquitin by isopeptidase in a situation analogous to that of H2A. Upon heat shock, denatured proteins would accumulate, acting as a sink for free ubiquitin in the cell. As with H2A, this would shift the equilibrium in favour of non-ubiquitinated HSTF, which

would be active in promoting transcription of heat shock genes (Munro and Pelham, 1985).

In addition to transcriptional regulation of the heat shock response, there is also differential regulation of translation of mRNAs during heat shock. When polysomal RNA from heat-shocked *Drosophila* culture cells is translated in a cell-free system from control cells, normal cellular proteins are synthesized in addition to heat shock proteins, indicating that mRNAs present in normal cells are not degraded upon heat shock, but are merely not translated (Storti *et al.*, 1980; Kruger and Benecke, 1981). In the converse experiment, polysomal RNA from control cells is not translated as efficiently as heat shock RNA in lysates from heat shocked cells.

Cells must therefore be able to distinguish between control and heat shock mRNA molecules. It has been reported that one discrimination mechanism involves the encoding of recognition information in the 5' untranslated leader region of hs mRNAs (McGarry and Lindquist, 1985). In this report, various deletions were introduced into *Drosophila* hsp70 genes that had been shortened to allow easy identification of their protein product, and *Drosophila* tissue culture cells were transformed with the modified genes. Deletions in the 5' untranslated leader that covered two conserved sequences prevented those mRNAs from being translated at heat shock temperatures. Upon return of the cells to ambient temperature, the modified hsp70 mRNAs were translated efficiently. This suggested that the two conserved sequences were responsible for the selective translation of hs messengers during heat shock.

Even with such a signal present in hs mRNAs, there would have to be modifications to the translational machinery to allow it to recognize such a signal, since the protein synthetic machinery from control cells translates control and hs mRNA with equal efficiency (Kruger and Benecke, 1981). Such modifications have not yet been satisfactorily explained.

In *Drosophila*, certain heat shock genes have been shown to be expressed during

development in the absence of heat shock. Two mRNAs transcribed from the gene cluster containing the hsp28, hsp26, hsp23, and hsp22 genes were found to accumulate in late larval and pupal stages, but not previous or subsequent to those stages (Sirotkin and Davidson, 1982). Also, mRNAs encoding hsp83, hsp28 and hsp26 were shown to accumulate in nurse cells within adult ovaries and subsequently pass into oocytes (Zimmerman *et al.*, 1983). These results suggest the existence of multiple regulatory elements governing the expression of these genes. It has recently been demonstrated by transforming deletion variants of the hsp26 gene into germ line cells that there are separate regions in the promoter required for developmental and heat-inducible expression of the gene (Cohen and Meselson, 1985). It seems likely, therefore, that different *trans*-acting factors bind to different regulatory sites in order to activate transcription during development or heat shock. The developmental expression of various heat shock genes also implies a possible function for the proteins in normal embryonic development.

The ubiquity of the heat shock response in all species and the high degree of conservation of major hsps between species are suggestive of important functions for these proteins. As yet, however, little has been conclusively shown regarding specific functions for hsps.

The heat shock response appears to be involved in conferring thermotolerance (increased resistance to potentially lethal temperatures) on cells. Evidence for this follows several lines. First, it has been shown in *Drosophila* (Graziosi *et al.*, 1980) and in *Xenopus* (Heikkila *et al.*, 1985) that early embryos, which are not yet active in transcription (and therefore are not able to induce hs transcription), experience high mortality at heat shock temperatures. Once embryonic transcription has begun, the embryos are able to mount a heat shock response, and mortality during heat shock declines significantly. Secondly, some non-heat stresses that induce a heat shock response confer thermotolerance on Chinese

hamster fibroblasts (Li *et al.*, 1982). Finally, thermoresistant Chinese hamster fibroblast cell lines contain increased amounts of hsp70 and show enhanced induction of hsp synthesis after heat shock (Lazlo and Li, 1983). The manner in which hsps might confer thermotolerance on cells is not known.

Many studies have attempted to infer the specific functions of hsps from their intracellular locations during heat shock. These studies have been largely inconclusive, as the locations appear to vary depending on the heat shock conditions, the localization techniques used, and the investigators involved. For example, immunofluorescence studies using monoclonal antibodies to *Drosophila* hsp70 have shown that the protein concentrates in the nucleus upon heat shock and becomes distributed throughout the cytoplasm during recovery (Velaszquez and Lindquist, 1984). In addition, hsp70 has been shown to be a nucleic acid binding protein (DiDominico *et al.*, 1982), suggesting a nuclear function. Other immunolocalization studies, however, have shown hsp70 to be associated with intermediate filaments in the cytoskeleton in *Drosophila* (Falkner *et al.*, 1981) and with an integral membrane glycoprotein in mammalian and chicken cells (Hughes and August, 1982).

Two hsps have recently been conclusively identified in yeast. Hsp48 was purified chromatogaphically, and 23 amino acids at the N-terminal were sequenced (Iida and Yahara, 1985). When the sequence was compared to other yeast polypeptide amino acid sequences, the hsp48 sequence was found to be identical to that of the glycolytic enzyme enolase. Hsp48 was subsequently found to have enolase activity. More recently, yeast hsp35 has been identified as being another glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (S. Lindquist, unpublished results).

It has also been suggested recently that the four small hsps in *Drosophila* are cytoskeletal proteins (Leicht *et al.*, 1986). This is based on results demonstrating that monoclonal antibodies against the small hsps and against vimentin-like intermediate filament

proteins stain the same structures in heat-shocked Kc cells.

As stated previously, inducible synthesis and the synthesis of specific mRNAs and proteins have not been analyzed in *Xenopus laevis* erythroid cells. The heat shock response has, however, been studied in erythroid cells from other species. Erythrocytes from quail and frog exhibit heat-inducible synthesis of heat shock proteins at elevated temperatures (Atkinson and Dean, 1985). Quail erythrocytes synthesize hsps of 90, 70, and 26 kD, whereas frog erythrocytes synthesize only the 90 kD and 70kD hsps. Erythroid cells from anemic quail show the same pattern of hsp synthesis as normal quail. When quail erythrocytes are treated with actinomycin D, the heat shock response is repressed, suggesting that regulation of the response in these cells occurs at the transcriptional level, although the actual transcription of the individual genes in question was not examined.

Heat-inducible synthesis of hsp70 has also been detected in chicken reticulocytes (Morimoto and Fodor, 1984; Banerji *et al.*, 1984), which are at the preterminal stage of erythroid differentiation and therefore are more synthetically active than erythrocytes. Actinomycin D was found to inhibit the heat shock response in these cells as well, again suggesting transcriptional regulation (Banerji *et al.*, 1984). Northern blot analysis, however, revealed no increased accumulation of hsp70 mRNA during heat shock. *In vitro* runoff transcription was used to assay directly the transcriptional activity of the hsp70 gene. No increase in transcription of the gene was observed at heat shock temperatures, leading the authors to conclude that although transcription is required for the heat shock response in chicken reticulocytes, regulation of the response occurs at the translational level.

Nuclear runoff transcription, as used by Banerji and her co-workers, is an extremely useful technique for directly analyzing the transcriptional activities of specific genes. The technique, which was first developed in order to characterize nuclear RNA synthesized in tumor cells (Takahashi *et al.*, 1963), involves isolating nuclei, then incubating the nuclei in

the presence of nucleotide triphosphates to promote the synthesis of new RNA, which can then be purified and analyzed. A further refinement of this technique, in which ^{3}H-cytidine was incorporated into the newly synthesized RNA and then hybridized in solution to a known quantity of a specific DNA sequence (in this case rDNA), allowed determination of the proportion of total RNA comprised by a specific RNA species (18S and 28S rRNA) (Reeder and Roeder, 1972). Incorporation of radioactive precursors into *in vitro* transcribed RNA has been monitored and observed to level off after 30-40 minutes. The point at which incorporation reaches a plateau has been found to vary linearly with the concentration of nuclei in the reaction (Hadjiolov and Milchev, 1974). This result, along with the observation that inhibitors of transcription initiation such as heparin have no effect on the kinetics of RNA synthesis, led to the conclusion that the RNA synthesis in isolated nuclei consists of elongation of previously-initiated transcripts, with no new initiation events occurring. Thus, characterization of RNA by this method provides information regarding the relative number of transcriptional events that had been initiated on a specific gene (and therefore the relative transcriptional activity of that gene) at the time of nuclear isolation.

The present study involves an analysis of changes in constitutive and inducible synthesis of RNA and protein during erythropoiesis of *Xenopus laevis*. Protein synthesis was examined under ambient and heat shock conditions at each stage of erythroid development. Results indicate that erythroid differentiation is accompanied by changes in the pattern of both constitutive and inducible protein synthesis. The heat shock response is gradually repressed during differentiation, although the ability to mount a heat shock response is never completely lost. Mature erythrocytes are capable of synthesizing one hsp, hsp70, in response to heat shock.

Transcription in erythroid cells was analyzed using an *in vitro* nuclear runoff

transcription assay. The array of genes analyzed included a gene that is inducible (the hsp70 gene), genes that are constitutively expressed in most cell types (the actin and ribosomal genes), and genes that are expressed in a tissue-specific manner (the globin, epidermal keratin, and vitellogenin genes). The data indicate that transcription of the hsp70, ribosomal, actin, and globin genes is gradually repressed during erythropoiesis. In spite of this repression, the ribosomal genes are transcribed constitutively in mature erythrocytes, and transcription of the hsp70 and actin genes is inducible in erythrocytes by heat shock. In addition, a keratin-like gene is transcribed constitutively in erythrocytes, and this transcription is increased during heat shock.

These results suggest that although there is a general repression of synthesis during *Xenopus* erythroid differentiation, terminally differentiated erythrocytes retain significant actual and potential synthetic activity, indicating that these differentiated cells are not as quiescent as has been believed.

MATERIALS AND METHODS

A) Chemicals and Reagents

All chemicals, unless otherwise specified, were purchased from either Fisher Scientific or Sigma Chemical Company. ^{35}S-methionine and uridine 5'-[α-^{32}P] -triphosphate (^{32}P-UTP) were obtained from Amersham.

B) Induction of Anemia

Adult male *Xenopus laevis* purchased from Nasco International were used for all experiments. Frogs were made anemic by administration of 0.5 ml of 0.5% phenylhydrazine HCl in *Xenopus* Ringer solution (88mM NaCl, 1 mM KCl, 15mM Tris, pH 7.6) in two dorsal subcutaneous injections on two successive days (Thomas and Maclean, 1975).

C) Collection and Washing of Blood

Before blood collection, normal and anemic frogs were anaesthetized by placing the animals in a 0.5% solution of ethyl m-aminobenzoate (Tricaine methanesulfonate) in tap water. The frogs were bled by making an incision in the chest, exposing the heart, and making an incision in the ventricle. Blood forced out through the wound was collected in heparinized polypropylene tubes. The blood was centrifuged at 1000 x g, 4^{o}C for 10 minutes, and the buffy coat (which contains leukocytes and platelets) on top of the red cell pellet was removed with a Pasteur pipette (Leonard, 1980). The blood was then washed twice by suspending the cells in 4 ml of *Xenopus* Ringer solution, centrifuging as stated above, and removing all but the erythroid cell pellet with a Pasteur pipette. For protein synthesis studies, the final cell pellet was resuspended in 1.0 ml of *Xenopus* Ringer solution and 1.0 ml of minimal essential medium (MEM - Flow Laboratories) lacking glutamine and methionine. For RNA studies, the pellet was resuspended in 1.0 ml of MEM. Homogeneity of erythroid cell preparations was verified by microscopic examination of cell smears stained

with Wright's stain (0.3% w/v of xanthine and thiazine in methanol). Cell concentrations were determined for each preparation using a Levy chamber hemacytometer.

D) Protein Synthesis Studies

Aliquots (typically 0.015 ml) of erythroid cell suspensions were incubated in microfuge tubes either at ambient temperature or at various heat shock temperatures in water-filled wells in Temp Blok heaters for 30 minutes. If constitutive protein synthesis were repressed by heat shock in *Xenopus* erythrocytes, this early pre-incubation would allow the repression to occur in heat-shocked samples prior to exposure of the cells to ^{35}S-methionine. This would serve to reduce background protein synthesis, facilitating interpretation of results. Samples were then incubated for a further 2.5 hours at the same temperature after the addition of 0.10 ml of MEM containing 0.080 mCi of ^{35}S-methionine. All samples were incubated for an additional 30 minutes at room temperature (Morimoto and Fodor, 1984). This final incubation resulted in all cells being incubated at the appropriate temperature for 3 hours and being labeled for 3 hours. In experiments testing for the requirement of transcription for protein synthesis, actinomycin D was added to the cell suspensions to a final concentration of 10µg/ml from 1mg/ml stock prior to the initial 30 minute incubation.

Unincorporated ^{35}S-methionine was removed by washing the cells three times in *Xenopus* Ringer solution containing 0.025M cold methionine and centrifuging for 5 minutes at 100 x g, 4°C. The cells were then lysed in 0.2 ml of protein sample buffer (0.4 mM Tris, 37 mM glycine, 6M urea [BioRad], 1% sodium dodecyl sulfate [SDS - BioRad], and 2% ß-mercaptoethanol) and heated to 100°C for 5 minutes.

Incorporated radioactivity was measured as follows: A 0.005 ml aliquot of each cell lysate was dotted onto glass fiber filters, which were then placed on a Millipore suction filtration apparatus. Protein was precipitated by filtering approximately 10 ml each of 25%

trichloroacetic acid (TCA) with 0.025M methionine, 8% TCA with 0.025M methionine, and 95% ethanol through each filter. Filters were thoroughly dried in an oven at 80°C, and the acid-precipitable radioactivity was measured by placing each filter in 10 ml of toluene scintillation fluid (6% v/v of Spectrafluor - Amersham) in a scintillation vial and counting in a Rackbeta scintillation counter.

Newly synthesized proteins in each sample were analyzed using SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and fluorography. Equal acid-insoluble radioactivity was loaded into each sample well of a 7-13% polyacrylamide gradient gel, and proteins were separated by electrophoresis at 20mA. After electrophoresis, proteins were fixed by soaking the gels for 10 minutes each in three washes of prestain solution (9.2% acetic acid, 45.4% methanol), and the proteins were stained by soaking the gels for 15 minutes in Coomassie blue (0.625% in prestain solution), followed by destaining overnight in 1.5% methanol, 1% acetic acid. Destained gels were prepared for fluorography by soaking for 15 minutes in Enlightning (New England Nuclear). The gels were subsequently dried onto Whatman No.1 paper under vacuum at ambient temperature for 30 minutes, then under vacuum at 80°C for one hour (Heikkila *et al.*, 1985). Dried gels were then exposed to Kodak X-Omat film in light-tight cassettes with Chronex intensifying screens at -70°C for 2 to 8 weeks, depending on the amount of radioactivity loaded onto each gel. Resulting fluorograms were developed in a Pako automated X-ray film developer.

Individual fluorograms were scanned using a Joyce-Loebl Chromoscan 3 laser densitometer to allow direct comparison of the intensities of individual radioactive protein bands.

E) Protein Blotting

For protein blotting experiments, the protein concentration of each sample was determined using a BioRad protein assay. Equal amounts of protein were loaded into each

sample well of a 10% polyacrylamide gel and electrophoresed at 20mA. After electrophoresis, the gel was soaked for 30 minutes in transfer buffer (25mM Tris, pH 8.3, 192mM glycine, 20% v/v methanol). Protein was electroblotted (Bittner *et al.*, 1980) onto nitrocellulose (Schleicher and Schuell BA83) overnight at 300mA at 4°C using a BioRad Transphor unit. Reaction of the blot with antibody was done using the procedure of Dr. S. Lindquist (personal communication). The protein blot was blocked for 2.5 hours at room temperature in 5% non-fat dry milk in Tris-buffered saline (TBS- 20mM Tris, pH 7.5, 500mM NaCl). After blocking, the blot was rinsed briefly in TBS and placed with rat monoclonal anti-*Drosophila* hsp70 (antibody 7.10 [Kurtz *et al.*, 1986] - a gift from Dr. S. Lindquist) in TBS into a heat-sealed bag at room temperature for 2 hours. The blot was subsequently washed on a shaker for 10 minutes in 5% milk containing 0.05% Tween 80 (J.T. Baker Chemical Company) and for an additional 10 minutes in TBS. The blot was then reacted with a 1:50 dilution of the secondary antibody (rabbit anti-rat IgG- Cooper Biomedical) in TBS for one hour at room temperature in a sealed bag. The filter was washed as after the first antibody and reacted with a tertiary antibody (horseradish peroxidase-linked donkey anti-rabbit IgG - Amersham) diluted 1:50 in TBS. After a final set of washes done as before, antibody-bound protein bands were visualized by exposing the filter to 0.05% diaminobenzidine, 0.003% hydrogen peroxide in TBS for 10 minutes. As a control, duplicate protein blots were treated identically except that treatment with the primary antibody was omitted.

F) *In vitro* Transcription

In vitro transcription was performed using the methods of Banerji *et al.* (1984). For the transcription studies, 0.6 ml aliquots of an erythroid cell suspension containing 1-7 x 10^8 cells were incubated at ambient temperature or at heat shock temperatures for one hour in

autoclaved microfuge tubes. After incubation, cells were lysed by the addition of 0.25 ml of 10mM Tris (pH 7.4), 10mM NaCl, 3mM $MgCl_2$, 0.5% Nonidet P-40 (NP-40). The concentration of NP-40 was sufficient to lyse the cells without disrupting the nuclei, so the nuclei could be pelleted after cell lysis by centrifuging at 1000 x g for 5 minutes. The supernatant containing the cytoplasmic constituents was removed with a sterile Pasteur pipette, and the nuclear pellet was resuspended in 40% glycerol, 50mM Tris (pH 8.3), 5mM $MgCl_2$, 0.1mM EDTA to a total volume of 0.5 ml. Aliquots of 0.1 ml of nuclear suspension were stored at -70°C.

After the addition of 0.1 ml of transcription buffer (50mM HEPES, pH 7.5, 5mM $MgCl_2$, 2.5 mM dithiothreitol, 5% glycerol, 350μM ATP, 350μM CTP, 350μM GTP, and 0.4μM UTP), 100 units of ribonuclease inhibitor, and 200μCi of ^{32}P-UTP to each 0.1 ml aliquot of nuclei, runoff transcription was allowed to proceed for 20 minutes at room temperature in a total volume of 0.24 ml. For transcription inhibitor studies, α-amanitin was added to the nuclear suspension to a concentration of 5μg/ml and allowed to incubate at room temperature for 10 minutes before addition of the transcription buffer. Reactions were stopped by the addition of 20 units of RNAase-free DNAase I (Promega Biotec), followed by incubation for an additional 30 minutes at 37°C. Each transcription reaction mixture was then diluted to 2 ml with a buffer consisting of 2% SDS, 7M urea, 0.35M NaCl, 1mM EDTA, and 10mM Tris, pH 8. Each sample was then extracted once with an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1) containing 0.1% 8-hydroxyquinoline and once with chloroform/isoamyl alcohol alone. Ammonium acetate was then added to a concentration of 0.25M, and RNA was precipitated by the addition of 2.5 volumes of 95% ethanol overnight at -20°C. RNA was pelleted by centrifugation at 2500 x g, 0°C, for 45

minutes. RNA pellets were air-dried, then redissolved in 0.09 ml of sterile water.

To measure the incorporation of radioactive precursor into RNA, 0.002 ml of each RNA sample was dotted onto a glass fiber filter and precipitated with 10% TCA followed by 95% ethanol. The filters were dried and scintillation counted in 10 ml of scintillation fluid. To compensate for different amounts of nuclei in each original transcription mixture, the number of nuclei present in a sample was determined from the original cell concentration and used to calculate the total amount of DNA in the sample (assuming 3.15pg of DNA per haploid *Xenopus* genome - Dawid, 1965). Incorporation values were then standardized by expressing the values in terms of counts per minute (cpm) per 100µg of DNA.

G) Recombinant DNA Clones

The recombinant DNA clones used in this study included the *Xenopus laevis* hsp70 gene cloned into pUC 12 (pXl10 XP [Bienz, 1984] - a gift from Dr. M. Bienz), the *Xenopus* 18S and 28S ribosomal genes (one transcription unit) cloned into pBR322 (pXlr-101A [Bakken *et al.*, 1982] - a gift from Dr. R.H. Reeder), the *Xenopus* ß-globin gene cloned into pGEM-3 (pSP6-ßXm [Melton, 1985] - a gift from Dr. D.A. Melton), the *Xenopus* epidermal keratin gene cloned into pHC624 (pC8128 [Jonas *et al.*, 1985] - a gift from Dr. T. Sargent), *Xenopus* vitellogenin B1 cDNA cloned into pBR322 (pXlvc 10.1 [Wahli *et al.*, 1979] - a gift from Dr. W. Wahli), and the mouse actin gene cloned into pBR322 (plasmid 91 [Minty *et al.*, 1981] - a gift from Dr. A.J. Minty).

H) DNA Dotting and DNA-RNA Hybridization

To select specific sequences out of the general population of radioactively labeled RNA, the corresponding DNA sequence was dotted in excess onto nitrocellulose (DNA was dotted in excess to ensure the binding of all of the requisite RNA sequences in each sample). To ensure ten-fold excess of DNA, 5µg of each recombinant clone was dotted. In order to confirm that dotted DNA was in excess, some experiments were repeated using 2µg DNA

dots. Results observed for 2μg dots did not vary significantly from results for 5μg dots, indicating that DNA was in excess for both 2μg and 5μg dots. DNA samples were dotted in triplicate, and plasmid vector DNA (lacking insert) corresponding to each recombinant clone was also dotted in triplicate onto nitrcellulose as a negative control.

Dotting was performed as follows: DNA was diluted with sterile water to a volume of 0.50 ml per dot. The solution was made 0.3M with respect to NaOH and was heated to 65°C for 30 minutes to denature the DNA. The solution was then neutralized by the addition of an equal volume of 2M ammonium acetate (Kafatos *et al.*, 1979). The DNA was then applied to a Schleicher and Schuell Minifold 96-sample well dotting manifold and filtered under vacuum onto a nitrocellulose sheet that had been prewetted for 10 minutes in water, then for 10 minutes in 3 X SSC (1 X SSC: 0.15M NaCl, 0.015M sodium citrate, pH 7). The DNA sample wells were then washed through with 0.30 ml of 3 X SSC. After air drying, the nitrocellulose was baked in a vacuum oven at 80°C for 2 hours to bind the DNA to the filter.

Individual DNA dots were cut out and marked with a pencil for identification, and each recombinant DNA dot was placed, along with a corresponding control dot, into a 4 ml scintillation vial. Hybridization was carried out as outlined by Banerji *et al.* (1984). The dots were prehybridized at 42°C for at least 4 hours in 0.2 ml of a partial hybridization mix (50% formamide, 6 X SSC, 5 X Denhardt's solution [50 X Denhardt's: 1% bovine serum albumin, 1% Ficoll - Pharmacia, 1% polyvinyl pyrrolidone - Denhardt, 1966], 0.1% SDS, 0.10 mg/ml yeast tRNA - Boehringer Mannheim). After prehybridization, the partial hybridization mix was removed from each vial and replaced with 0.20 ml of hybridization mix (4 parts prehybridization mix plus 1 part 50% dextran sulfate) containing 10^5 to 10^7 cpm of labeled RNA (which had been denatured at 65°C for 15 minutes) from control or heat-shocked nuclei. Each hybridization reaction was then overlayed with mineral oil to prevent evaporation (Farnham and Schimke, 1985), and hybridization was carried out at

42°C for 48 to 72 hours. Blots were washed at 65°C for 30 minutes in each of three changes of 2 X SSC, 0.1% SDS. These washing conditions were calculated (using a formula outlined in Maniatis *et al.*, 1982) to allow only the association of RNA-DNA hybrids with greater than 90% homology. The blots were then air-dried and scintillation counted to determine the amount of labeled material hybridized to each dot. ^{32}P cpm bound to negative control dots were subtracted from cpm bound to corresponding filters containing recombinant plasmid. Values from triplicate dots were averaged, and the mean was standardized to cpm per 100μg of template DNA. Relative transcription level of each RNA species was calculated by dividing hybridized cpm by total cpm added to the hybridization mixture. This value was expressed in parts per million (ppm) (McKnight and Palmiter, 1979). Significance of variation between samples at the 0.05 level was determined using a Wilcoxon two-sample test.

RESULTS

1. Effects of Heat Shock on Erythrocyte Protein Synthesis

To determine the effects of elevated temperatures on protein synthesis in mature *Xenopus* erythrocytes, newly-synthesized proteins were labeled with ^{35}S-methionine at ambient temperature or at one of various heat shock temperatures. Proteins were separated electrophoretically, and the labeled proteins were visualized by fluorography. The resulting fluorograph is shown in Figure 1, and densitometer scans of the individual fluorograph lanes are shown in Figure 2. It is apparent from these figures that *Xenopus* erythrocytes are engaged in constitutive protein synthesis. Globin is the major protein synthesized, supporting the conclusions of Maclean *et al.* (1969), but many other proteins are synthesized as well. Although increased temperatures cause a decrease in the synthesis of most proteins, the synthesis of a 70kD protein (hsp70) is strongly induced at 33°C, 10° above ambient. Synthesis of this protein is induced over a very narrow range of temperatures; it is synthesized slightly at 30°C and not at all at 35°C or 37°C. This is in contrast to the results seen for erythrocytes of other frog species, which synthesize hsp70 at 37°C (Atkinson and Dean, 1985). In addition, a set of apparently constitutively synthesized proteins was observed whose synthesis is not repressed by heat shock. Examination of densitometer scans reveals that the intensities of these protein bands (with M_r of 61kD, 43kD, 32kD, 30kD, and 28kD) are increased at higher heat shock temperatures. This increase may be artefactual, however, because overall protein synthesis is repressed at higher temperatures, so incorporation of labeled methionine is reduced in the higher temperature samples. Thus, in order to equalize the radioactivity loaded in each gel lane, more total protein from the 35°C and 37°C samples had to be loaded into their respective lanes. As a result, if each of these proteins was present at equal concentrations in every sample, more of these heat-resistant

Figure 1. Fluorograph of proteins labeled with ^{35}S-methionine in *Xenopus* erythrocytes at ambient temperature (A) and at heat shock temperatures of 30°C, 33°C, 35°C, and 37°C. Hsp70 is indicated by an open arrowhead, and heat shock-tolerant proteins are indicated with solid arrowheads.

ERYTHROCYTES

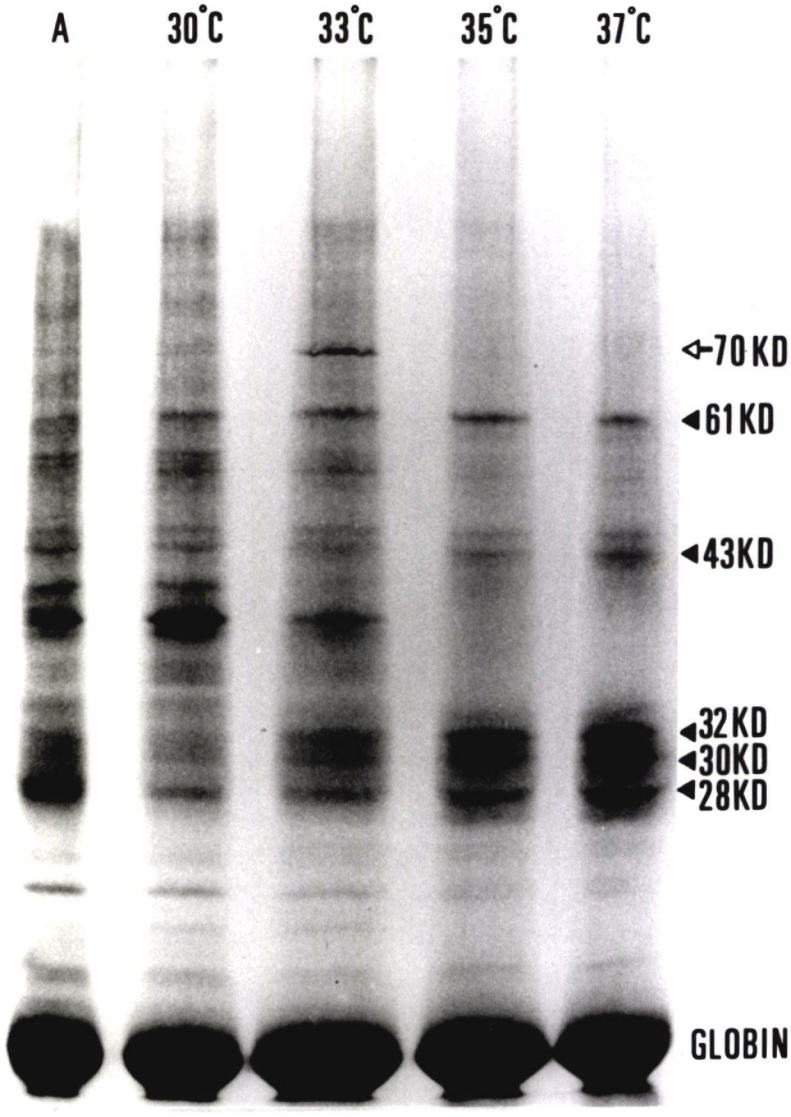
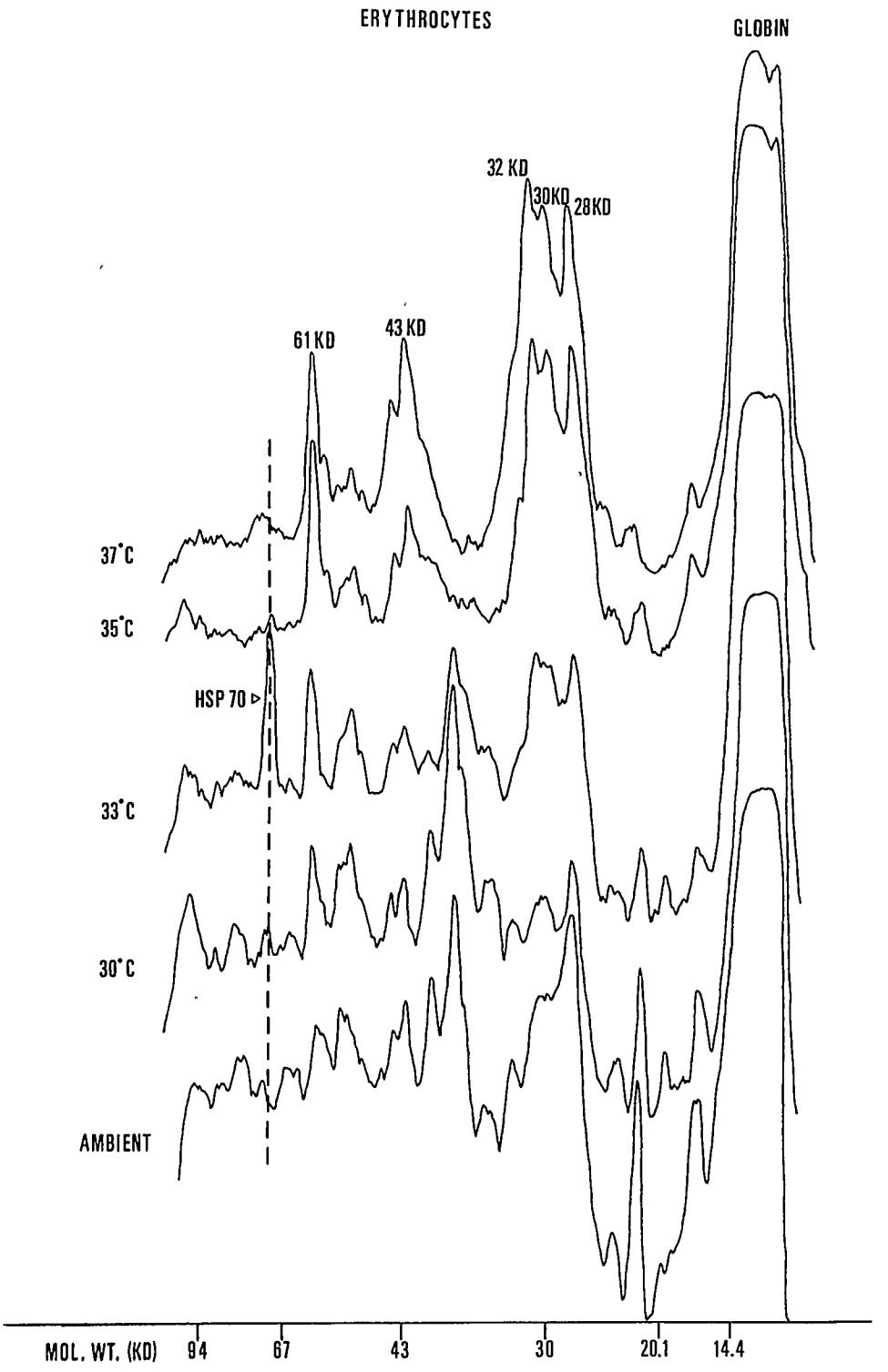


Figure 2. Densitometer scans of the individual fluorograph lanes in Figure 1. The 70kD position is indicated by a dotted line. The peaks representing heat shock-tolerant proteins have been indicated by their molecular weights.


ERYTHROCYTES
GLOBIN
32 KD
30KD
28KD
61 KD
43 KD
37°C
35°C
HSP 70
33°C
30°C
AMBIENT
MOL. WT. (KD)
94
67
43
30
20.1
14.4

proteins would be loaded into the higher temperature heat shock lanes than the other lanes. For this reason, these proteins have been classified as heat shock-tolerant proteins, rather than heat shock proteins. Because hsp70 synthesis is maximal at 33°C, all subsequent heat shocks were performed at that temperature.

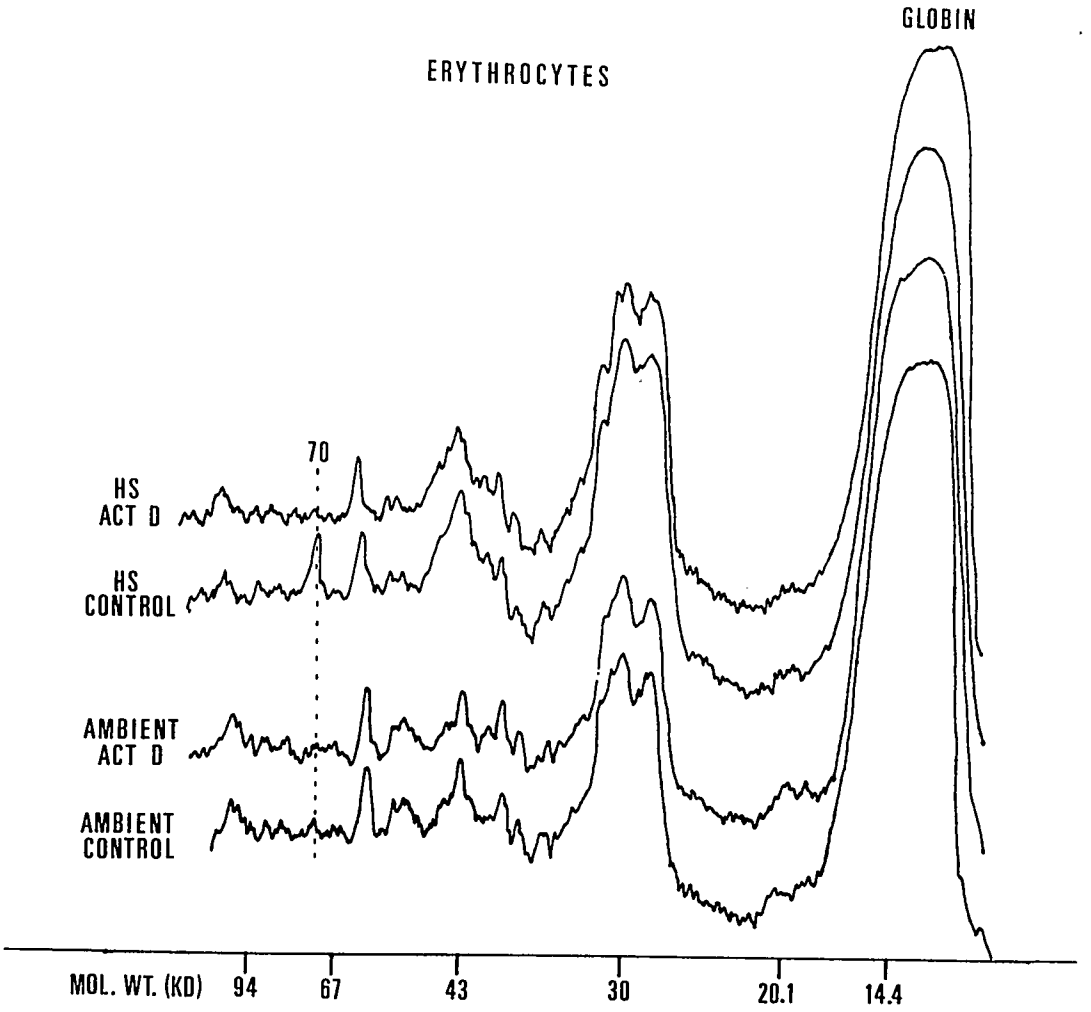
To test for the requirement of transcription for protein synthesis at ambient and heat shock temperatures, erythrocytes were incubated at room temperature and at 33°C with the transcription inhibitor actinomycin D added to a concentration of 10 μg/ml 10 minutes before incubation at the appropriate temperature. The densitometer scans of the fluorograph from this experiment (Figure 3) show that actinomycin D at this concentration (which should completely inhibit transcription) does not affect constitutive protein synthesis in erythrocytes. This suggests that under normal circumstances, protein synthesis in these cells utilizes previously synthesized mRNA, as was suggested by Maclean *et al.* (1973). In contrast, synthesis of hsp70 by heat-shocked erythrocytes is inhibited by actinomycin D, implying that rather than using stored mRNA, hsp70 synthesis is dependent upon *de novo* transcription. An alternative interpretation is that actinomycin D selectively inhibits the translation of hsp70 mRNA. The latter possibility seems unlikely, but direct analyses of transcription in erythrocytes are required to choose conclusively between the alternative explanations and to determine which genes, if any, are being transcribed during heat shock. The results of those analyses are presented in Section 3.

2. Effects of Heat Shock on Erythroblast Protein Synthesis

A. Time Course of Recovery From Anemia

Recovery of *Xenopus* from phenylhydrazine-induced anemia was characterized by analyzing the composition of the erythroid cell populations at various times after phenylhydrazine injection. The proportion of the total erythroid population comprised of each

Figure 3. Densitometer scans of fluorograph lanes of erythrocyte proteins labeled with ^{35}S-methionine in the presence (ACT D) or absence (CONTROL) of 10μg/ml actinomycin D at ambient temperature or at a heat shock (HS) temperature of 33^{o}C. 70kD is indicated by a dotted line. Note the 70kD protein peak in the HS CONTROL sample, which is absent from the sample treated with actinomycin D.


GLOBIN
ERYTHROCYTES
70
HS
ACT D
HS
CONTROL
AMBIENT
ACT D
AMBIENT
CONTROL
MOL. WT. (KD)
94
67
43
30
20.1
14.4

cell type was determined by microscopic examination of Wright's-stained smears of washed erythroid cell suspensions (Table 1). Erythroid cells were classified according to morphology and staining characteristics.

The erythrocyte content of blood was seen to decrease dramatically after administration of phenylhydrazine; within 16 days of the last injection, all but a small proportion of the mature cells had been lysed. Erythrocytes remaining in the circulation were very fragile, and there was a considerable degree of lysis while washing the cells. The course of erythrocyte destruction corresponds very well with that observed by Thomas and Maclean (1975).

The first basophilic erythroblasts were seen to appear in the peripheral circulation approximately 6 days after the final injection of the hemolytic agent. This is considerably earlier than the 15 days reported previously (Thomas and Maclean, 1975). This difference may be due to variations between populations of frogs, as well as to differences in diet and environment. After the appearance of the basophilic erythroblasts, differentiation was observed to proceed very synchronously, a phenomenon that has been reported for newts (Grasso and Shephard, 1968), but not for *Xenopus*. By 8 to 12 days after induction of anemia, polychromatophilic erythroblasts comprise a large majority of the immature erythroid cell population, whereas basophilic erythroblasts are now present only in small numbers. By 12 to 20 days after induction, orthochromatic erythroblasts make up virtually the entire erythroid population. Basophilic erythroblasts are no longer seen in the circulation, and only a few polychromatophilic erythroblasts have not yet differentiated.

As a result of the synchrony of differentiation, it is possible to obtain fairly homogeneous suspensions of each erythroblast type. The only major contaminating cell type after washing is the erythrocyte, and this fact was taken into account when interpreting results from erythroblasts. Because the erythroblast populations were not completely

CELL TYPE	DAYS AFTER PHENYLHYDRAZINE INJECTION		
	6 to 8	8 to 12	12 to 20
Erythrocytes	69.5	57.3	0.8
Basophilic Erythroblasts	30.5	2.2	1.4
Polychro-matophilic Erythroblasts	____	40.5	6.7
Ortho-chromatic Erythroblasts	____	____	91.1

Table 1. Percentage of each erythroid cell type in *Xenopus* erythroid populations at different times after injection with phenylhydrazine.

homogeneous, they will be referred to as being basophilic-enriched, polychromatophilic-enriched, or orthochromatic-enriched, based upon the major cell type present.

B. Protein Synthetic Patterns

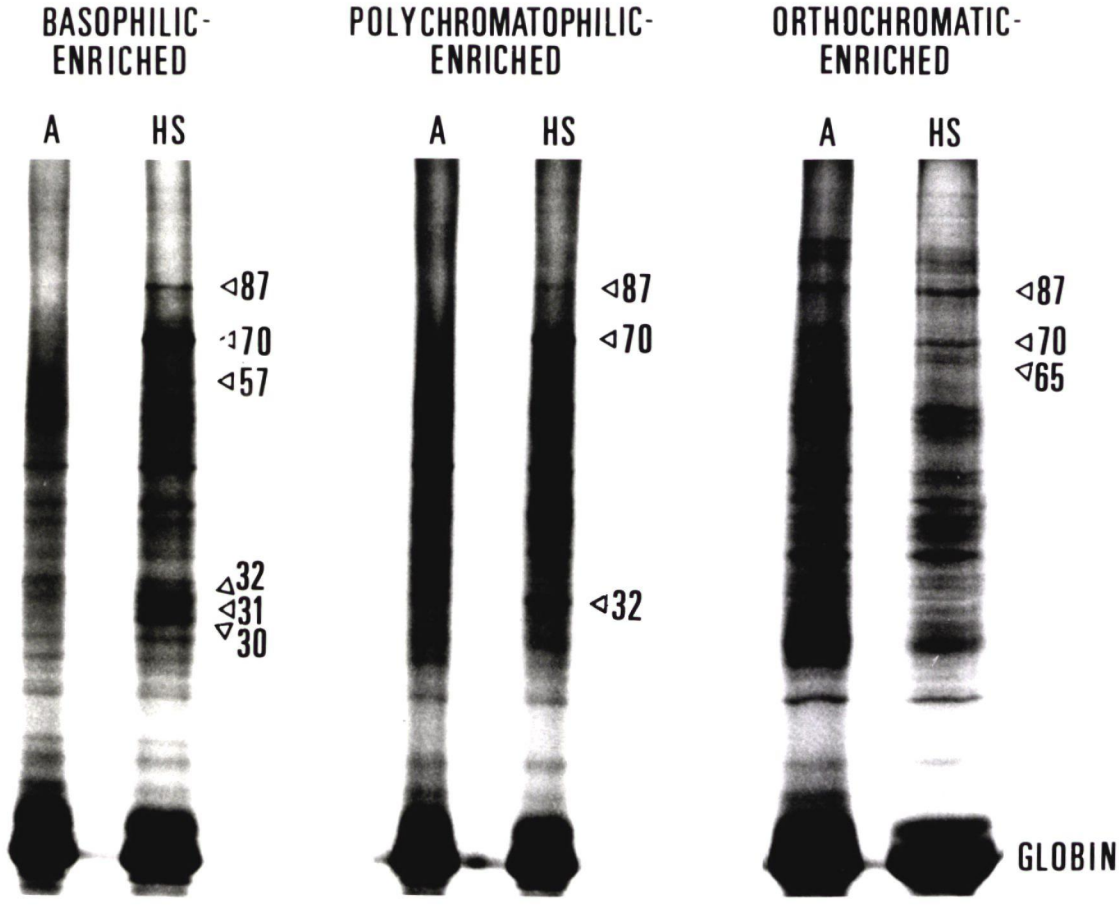
Suspensions of each erythroblast cell type were prepared and incubated at ambient or heat shock temperature in the presence of ^{35}S-methionine. Protein samples were prepared, and newly-synthesized proteins were analyzed as with erythrocytes. The resulting fluorographs are shown in Figure 4, and densitometer scans of the various fluorograph lanes are shown in Figures 5 to 7. The fluorographs show each erythroblast preparation to have its own characteristic pattern of protein synthesis, indicating that the protein profiles are due primarily to the unique contribution of the dominant erythroblast cell type and not to either residual erythrocytes or minor erythroblast constituents.

In the basophilic-enriched sample, heat shock induces the synthesis of hsps with M_r of 87kD (hsp87), 70kD (hsp70), 57kD (hsp57), 32kD (hsp32), 31 kD (hsp31) and 30kD (hsp30). All of these hsps have been previously identified in *Xenopus* (Heikkila *et al.*, 1985; Bienz, 1984), except for hsp32 and hsp31.

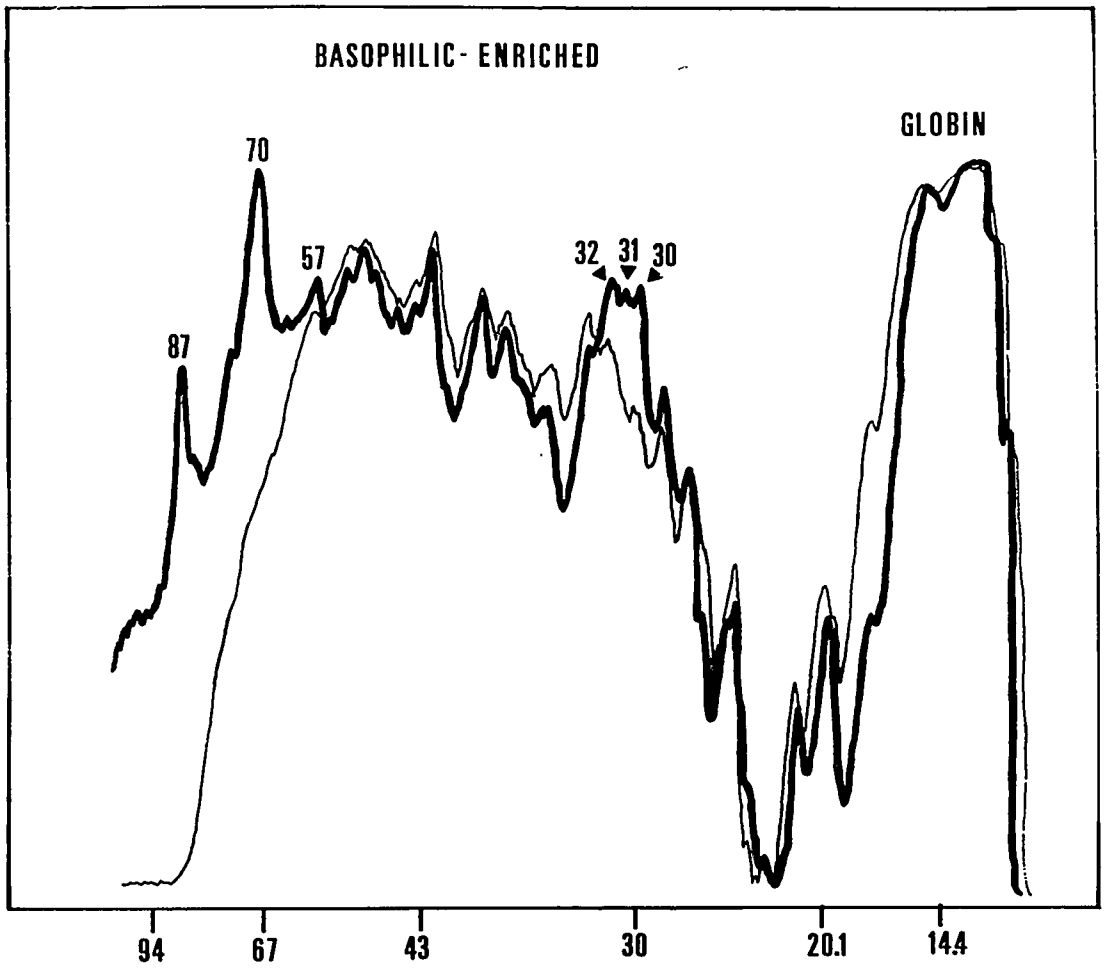
The polychromatophilic-enriched sample shows heat shock induction of hsp87, hsp70, and hsp32. Comparison of the densitometer scans of this sample (Figure 6) to those of the basophilic-enriched sample (Figure 5) reveals that the synthesis of each of these proteins is less in the later stage than in the earlier one. No induction of hsp57, hsp31, or hsp30 synthesis was observed in the polychromatophilic erythroblasts.

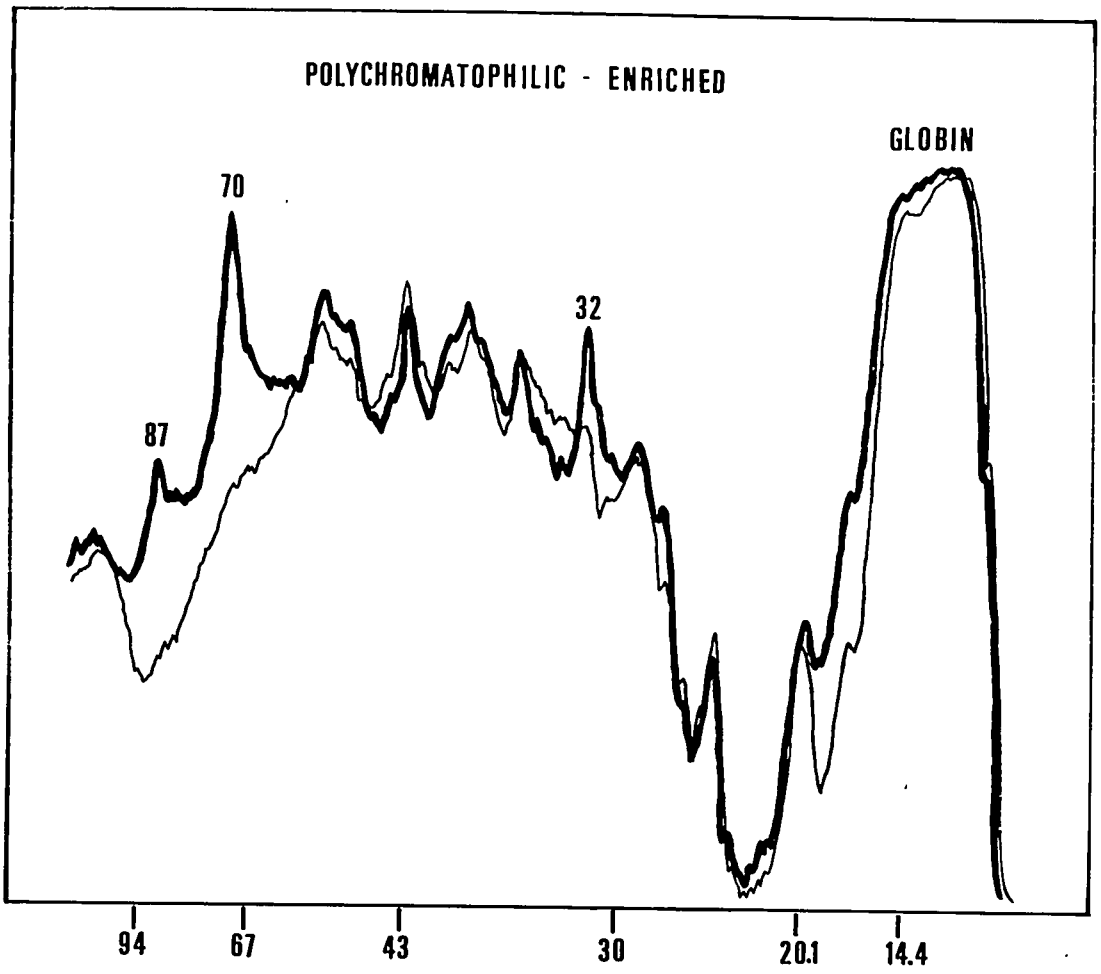
In the orthochromatic-enriched sample, synthesis of an 87kD protein and a 70 kD protein is seen at ambient temperature. These protein bands correspond exactly to the hsp87

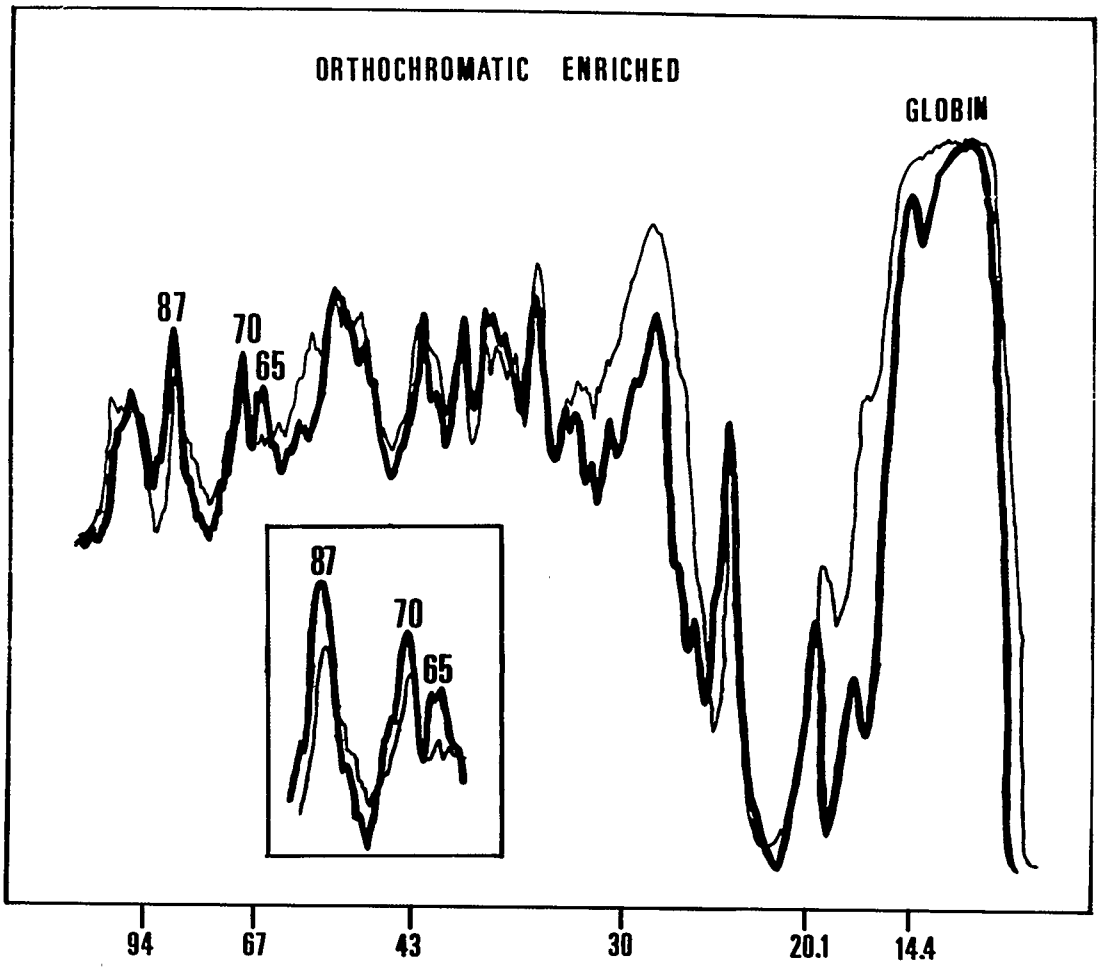
Figure 4.Fluorographs of proteins labeled with ^{35}S-methionine under ambient (A) and heat shock (HS) conditions in erythroid populations enriched in each erythroblast cell type. Hsps are indicated by open arrowheads.


BASOPHILIC-
ENRICHED
A
HS
87
70
57
32
31
30
POLYCHROMATOPHILIC-
ENRICHED
A
HS
87
70
32
ORTHOCHROMATIC-
ENRICHED
A
HS
87
70
65
GLOBIN

Figures 5,6, and 7. Densitometer scans of the fluorograph lanes from figure 4. Figure 5 consists of the scans of the basophilic-enriched lanes, Figure 6 consists of the scans of the polychromatophilic-enriched lanes, and Figure 7 consists of the scans of the orthochromatic-enriched lanes. In each figure, the scan of the ambient sample is represented by a narrow line, and the scan of the heat shock sample is represented by a bold line. Heat shock protein peaks are labeled by size. The inset in Figure 7 is an enlargement of one area of the figure, which demonstrates increased synthesis of hsp87 and hsp70 during heat shock over the levels of constitutive synthesis of the 87kD and 70kD proteins.


BASOPHILIC- ENRICHED
GLOBIN
70
57
87
32
31
30
94
67
43
30
20.1
14.4


POLYCHROMATOPHILIC - ENRICHED
GLOBIN
70
32
87
94
67
43
30
20.1
14.4


ORTHOCHROMATIC ENRICHED
GLOBIN
87
70
65
87
70
65
94
67
43
30
20.1
14.4

and hsp70 bands in the heat-shocked sample (Figure 7, inset). Levels of hsp87 and hsp70 synthesis during heat shock are slightly above the levels of the corresponding constitutive proteins. Treatment of orthochromatic erythroblasts with actinomycin D reduces hsp87 and hsp70 synthesis to the constitutive levels (Figure 8). These data suggest that the constitutively-synthesized proteins may be hsp70 and hsp87, but the data are insufficient to determine whether this is true or if the constitutive proteins are proteins with different identities from the hsps but identical mobilities in polyacrylamide gels.

To differentiate between these possibilities for the 70kD protein, immunodetection of hsp70 was used. Proteins from ambient and heat-shocked orthochromatic-enriched erythroid preparations were separated on a polyacrylamide gel and electroblotted onto nitrocellulose. The resulting protein blot was reacted with a monoclonal anti-*Drosophila* hsp70 antibody, which was visualized by immunoperoxidase staining (Figure 9). A control blot consisting of the same samples, but treated only with the secondary and tertiary antibodies, was also done. The major band seen on the test blot, but not on the control, was a 70kD band that appeared with approximately equal intensity in both the ambient and heat shock lanes. Proteins from ambient and heat-shocked erythrocytes were also blotted and treated with the monoclonal antibody. These samples show very low amounts of antibody-reactive protein in the ambient sample and an intense 70kD band in the heat shock lane. These data suggest that the constitutive 70kD protein synthesized in orthochromatic erythroblasts is an hsp70-like protein. Whether it is hsp70 itself or a heat shock cognate protein of similar size and structure has not been determined. As yet, however, no hs cognate proteins have been identified in *Xenopus*. The 87kD protein remains uncharacterized, but based on the results for the 70kD protein, it is possible that it is an hsp87-like protein. This would have to be tested by sequence comparison, peptide mapping, or immunodetection.

Figure 8. Densitometer scans of fluorograph lanes of proteins labeled with ^{35}S-methionine in erythroid cell preparations enriched in orthochromatic erythroblasts. Labeling was done in the presence (ACT D) or absence (CONTROL) of 10μg/ml actinomycin D under both ambient and heat shock conditions. Treatment of heat-shocked samples with actinomycin D reduced the 87kD and 70kD peaks to ambient levels.

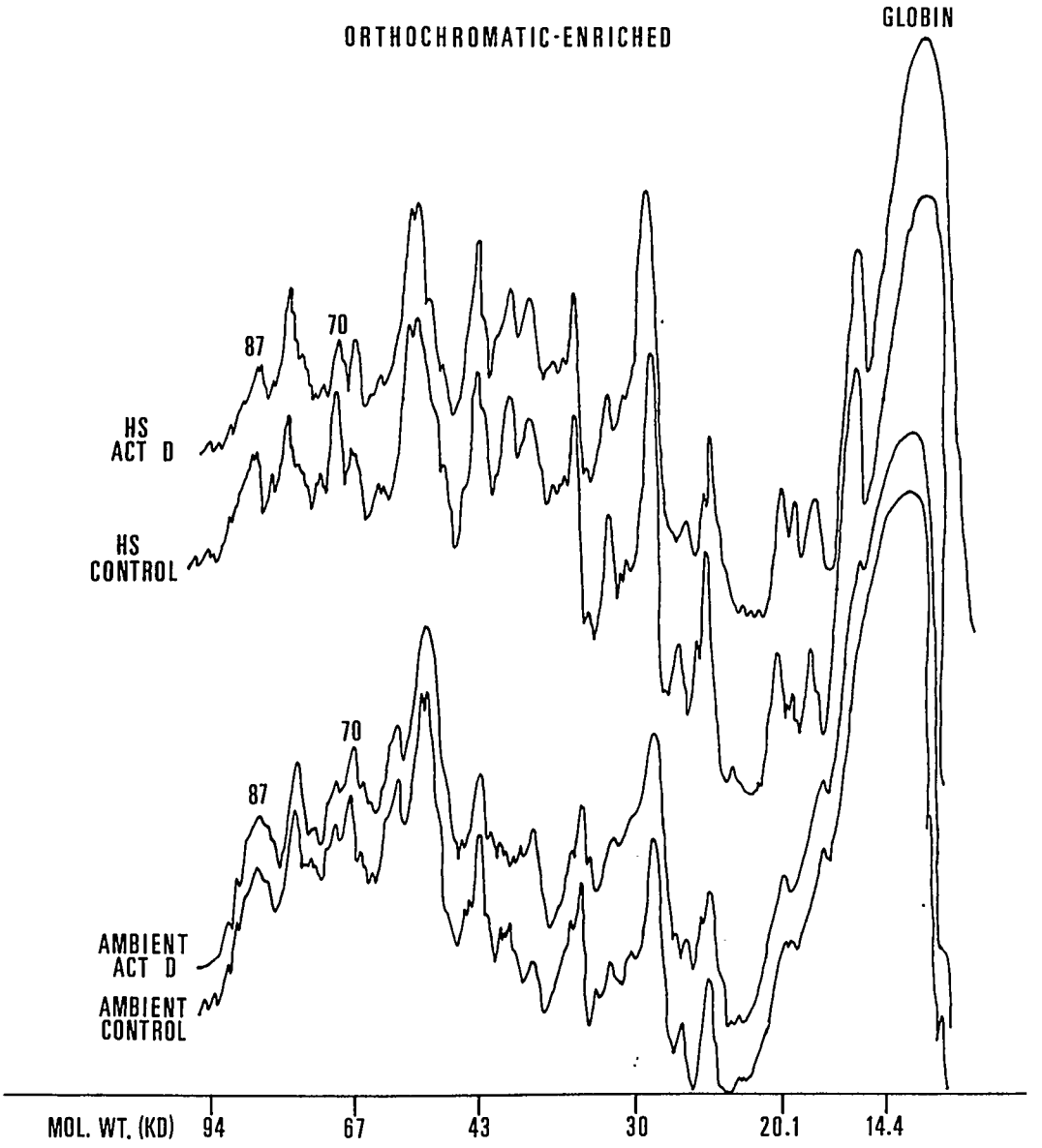
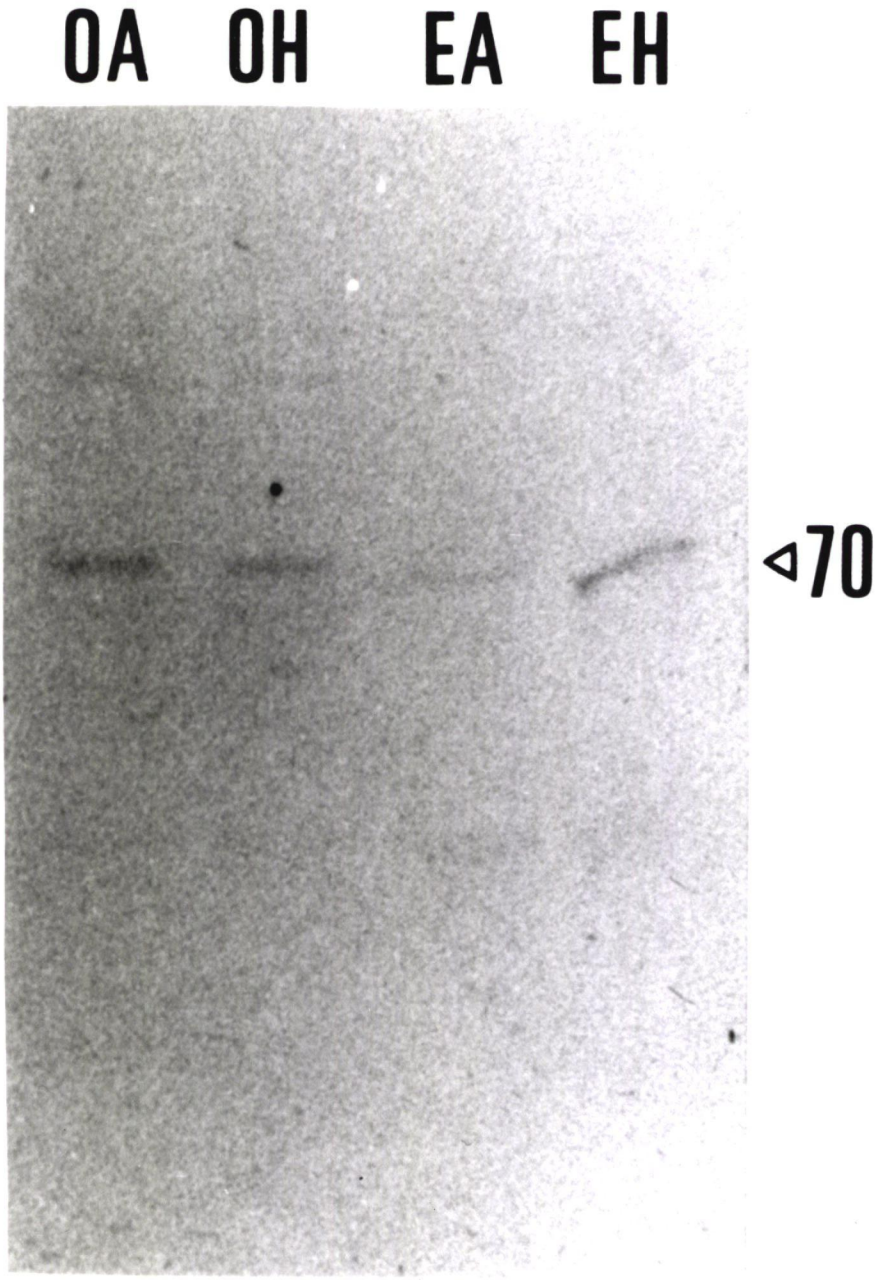

ORTHOCHROMATIC-ENRICHED
GLOBIN
87
70
HS
ACT D
HS
CONTROL
70
87
AMBIENT
ACT D
AMBIENT
CONTROL
MOL. WT. (KD)
94
67
43
30
20.1
14.4

Figure 9. Protein transfer blot treated with monoclonal anti-*Drosophila* hsp70 antibody and visualized by immunoperoxidase staining. Hsp70-like protein is present in both the orthochromatic-enriched ambient (OA) and heat-shocked (OH) samples in approximately equal amounts. Small amounts of the protein are detectable in ambient erythrocytes (EA), and greater accumulation of the protein is observed in heat-shocked erythrocytes (EH).


OA
OH
EA
EH
70

The orthochromatic-enriched preparation also shows heat shock-inducible synthesis of a 65kD protein that is not seen in any other erythroid cell type. An hsp of the same M_r has been observed at certain stages of *Xenopus* development, including artificially activated eggs (Pollock and Browder, unpublished results) and neurulae (Nickells and Browder, unpublished results). Hsp33 synthesis is no longer induced by heat shock in the orthochromatic-enriched sample.

The above data demonstrate that the heat shock response is gradually repressed during erythropoiesis, becoming restricted in the number of hsps synthesized and the levels of hsp synthesis. The heat shock response is never completely lost, however; even terminally-differentiated erythrocytes respond to heat shock with the induction of hsp70 synthesis. In addition, hsp70 synthesis in erythrocytes may require transcription to occur.

3. Analysis of Erythroid Transcription

Constitutive and inducible transcription were analyzed in erythrocytes and erythroblasts using a nuclear runoff transcription assay. In erythrocytes, the ribosomal and actin genes, which are constitutively transcribed in most cell types, and the globin gene, which is specifically expressed in erythroid cells, were tested to determine if any of these genes is normally active in the terminally differentiated cells. Inducible transcription in erythrocytes was examined by assessing transcriptional activity of the hsp70, actin, and keratin genes under ambient and heat shock conditions. The hsp70 gene was used because of the observation that heat shock-induced hsp70 synthesis in erythrocytes is inhibited by actinomycin D. Actin transcription during heat shock was assayed because actin mRNA has been shown to be more abundant in *Xenopus* embryos during heat shock (Heikkila *et al.*, 1985). Transcription of the keratin gene was examined in the belief that the gene would demonstrate no transcriptional activity in erythrocytes, because this gene has been reported to

be transcribed only in epidermal cells of *Xenopus* embryos (Jonas *et al.*, 1985).

For these assays, ^{32}P cpm hybridizable to the gene for vitellogenin, which is expressed only in hepatocytes (Wallace and Dumont, 1968), were used to determine the level of spurious transcription. Only hybridization levels significantly higher than vitellogenin hybridization levels were regarded as representing true transcription.

Transcription in the various erythroblast cell types was also assayed in order to follow changes in the activity of specific genes during erythropoiesis. Constitutive transcription levels of the ribosomal and globin genes were compared to corresponding levels in erythrocytes, as were levels of constitutive and inducible transcription of the hsp70 and actin genes. Such comparisons provide information regarding the RNA synthetic activity of erythrocytes relative to their more active precursors.

A. Constitutive Transcription in Erythrocytes

Transcription assays were performed by isolating erythrocyte nuclei and incorporating ^{32}P-UTP into runoff transcripts, which were then hybridized to specific cloned DNA sequences dotted onto nitrocellulose. Transcription assays were repeated several times; the results from a typical assay, expressed as absolute transcription levels (^{32}P cpm hybridized per 100μg of erythrocyte template DNA) are shown in Figure 10. Averages of data from all assays, expressed as the transcription level for each specific gene relative to total transcription (parts per million - ppm), are shown in Figure 11.

The results obtained for constitutive transcription in erythrocytes did not vary significantly from the expectation based upon the observations of Maclean *et al.* (1973). The ribosomal genes are constitutively transcribed at significant levels in erythrocytes. Radioactive cpm hybridizing to the ribosomal genes are more than one hundred-fold greater than for the other genes examined, supporting the observation of Maclean and his

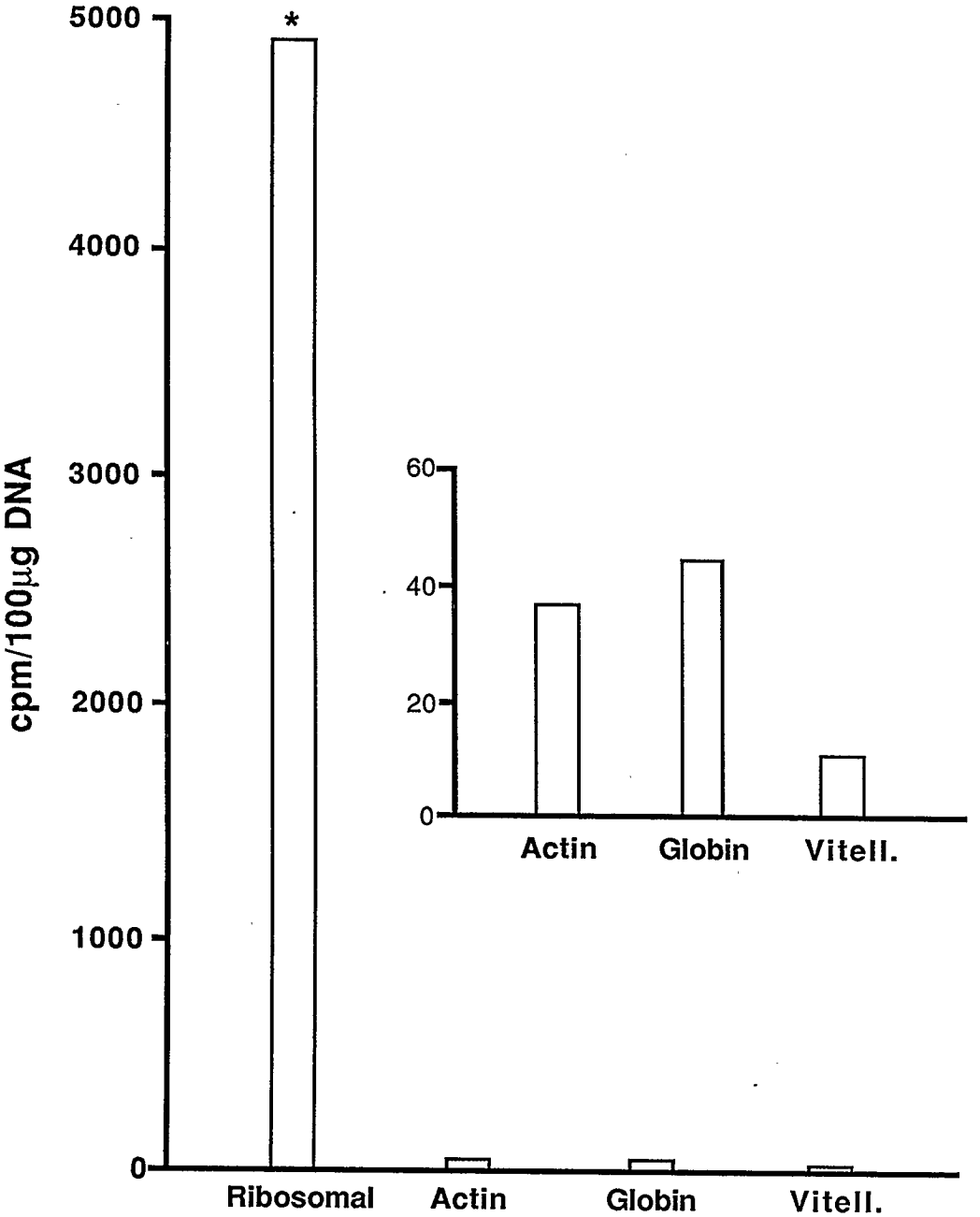


Figure 10. Typical assay of absolute constitutive transcription of the ribosomal, actin, ß-globin, and vitellogenin genes in erythrocyte nuclei. The transcription level is expressed as ^{32}P cpm/100µg of template DNA. Inset depicts the transcription levels for the actin, ß-globin, and vitellogenin genes using an expanded scale. Asterisk (*) indicates transcription that is significant at the 0.05 level compared to vitellogenin gene transcription.Actual cpm above background varied from 11.2 for vitellogenin to 6484 for ribosomal.

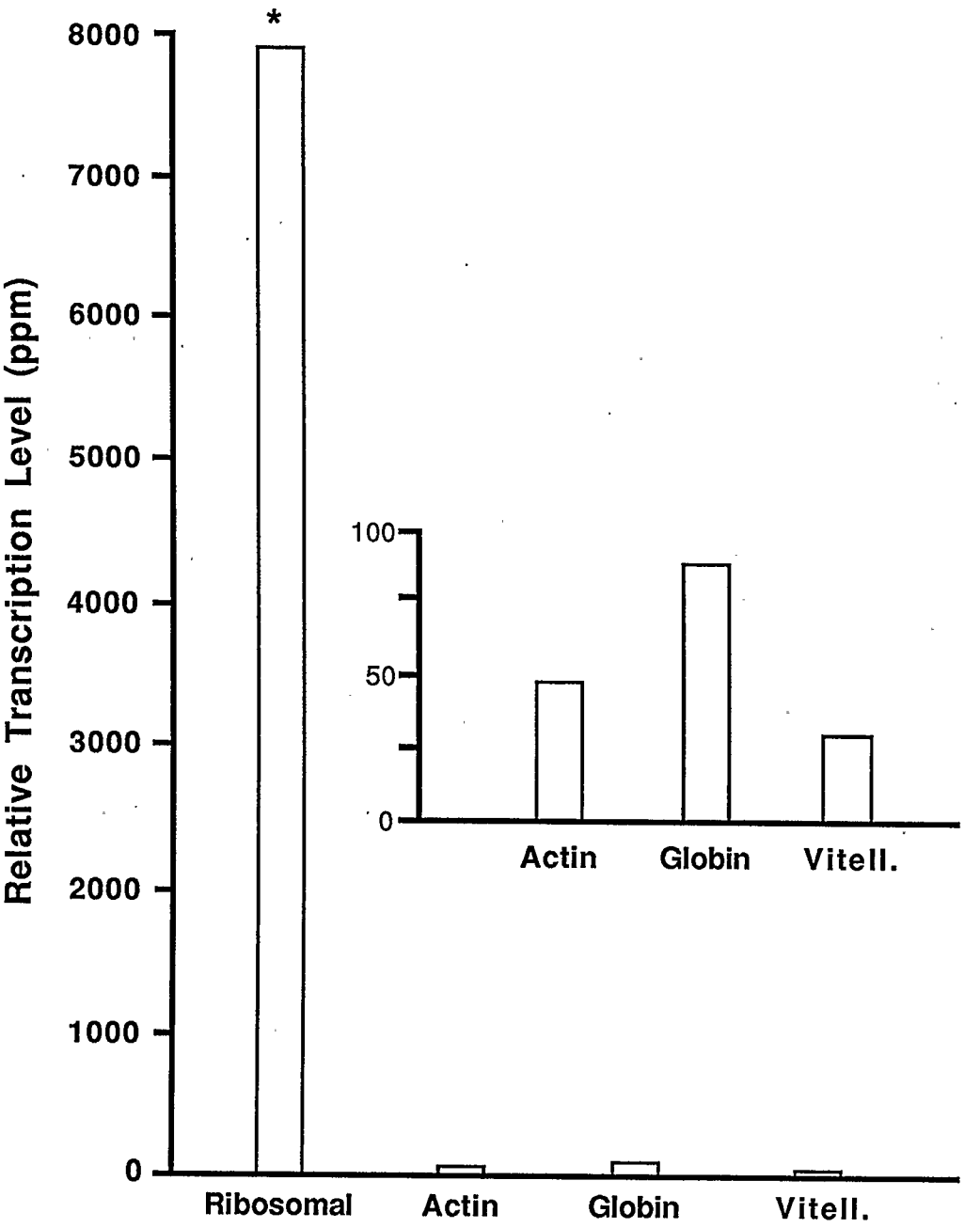


Figure 11. Constitutive transcription levels of the ribosomal, actin, ß-globin, and vitellogenin genes relative to total transcription in erythrocyte nuclei. Data on this figure represent means calculated from all assays performed. As in Figure 10, an * indicates transcription that is significant at the 0.05 level.

co-workers that ribosomal RNA is the major constituent of newly synthesized RNA.

Levels of transcription for the actin and globin genes were not significantly greater than spurious transcription as established with the vitellogenin gene, indicating that these two genes are transcriptionally quiescent in erythrocytes. The result for the globin gene supports an earlier result (Figure 3) showing that globin synthesis in *Xenopus* erythrocytes is resistant to actinomycin D, suggesting that globin synthesis utilizes stored mRNA synthesized at a previous stage.

B. Heat Shock-Inducible Transcription in Erythrocytes

For these experiments, ^{32}P-UTP was incorporated into runoff transcripts in nuclei isolated from ambient and heat-shocked cells. On average, incorporation of labeled UTP is 37% higher in nuclei from heat-shocked cells than those from ambient cells, indicating higher levels of transcription during heat shock. Results of the transcription assays are again presented both as cpm/100μg of template DNA (Figure 12) and as parts per million (Figure 13). As with the constitutive transcription assay, only hybridization levels significantly above those for the vitellogenin gene are considered to be true transcription.

The hsp70 gene shows no significant transcription in erythrocytes at ambient temperature. At 33°C, however, transcription of this gene increases dramatically. On average, the increase in relative transcription level was approximately ten-fold.This indicates that terminally-differentiated erythrocytes are capable of inducible transcription. Interestingly, actin gene transcription follows a similar pattern. As reported above, no significant transcription of actin genes is observed at ambient temperature. However, significant levels of transcription occur during heat shock.On average, the increase in actin transcription is two- to threefold, which is less than that for the hsp70 gene. The keratin gene, surprisingly, is transcribed constitutively in erythrocytes. The transcription levels of the gene are very low

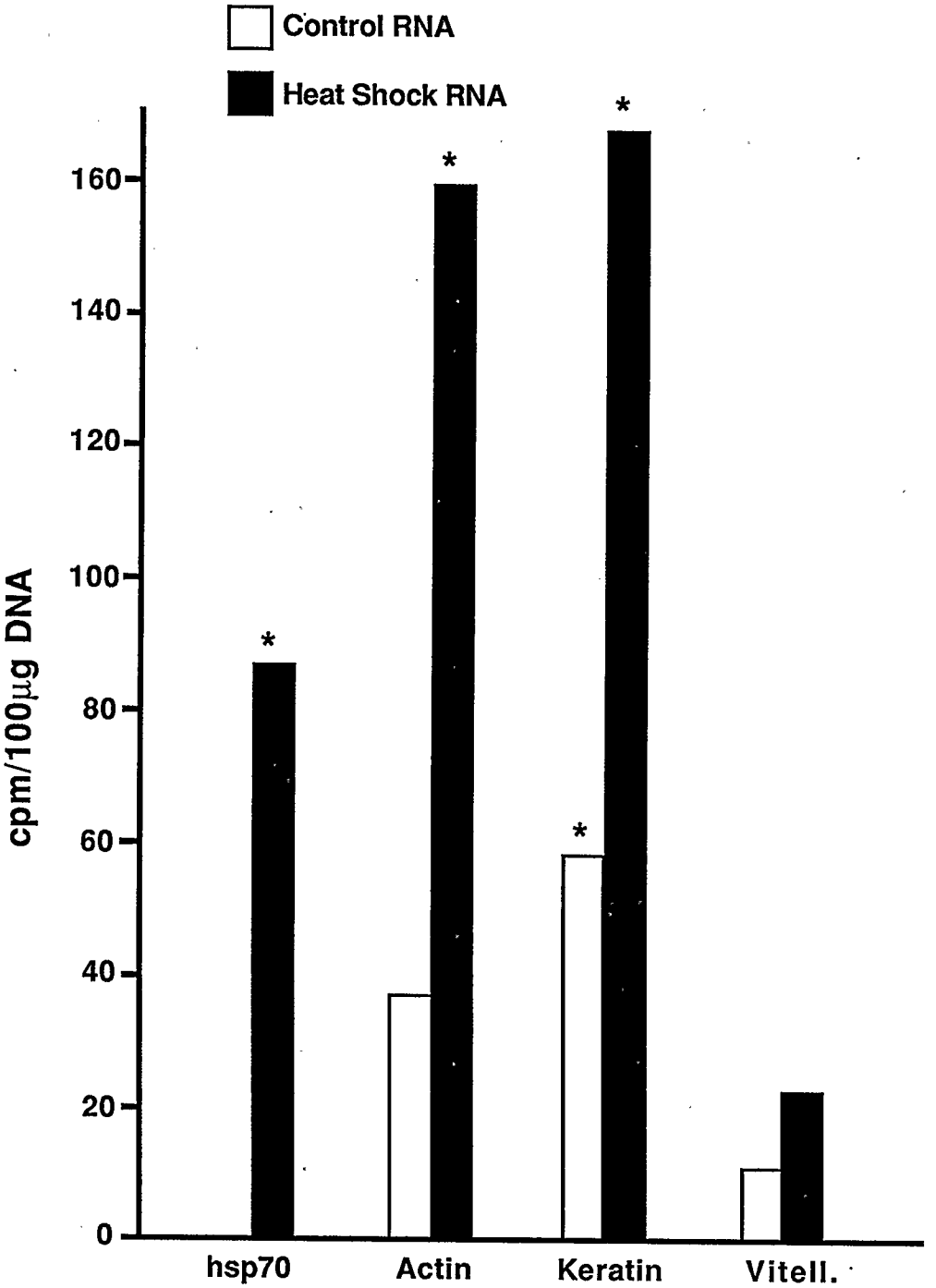


Figure 12. Typical assay of absolute transcription levels of the hsp70, actin, keratin, and vitellogenin genes in erythrocyte nuclei under ambient and heat shock conditions. Asterisks (*) indicates transcription which is significant at the 0.05 level compared to vitellogenin gene transcription. Actual cpm above background varied from 0 (hsp70) to 86.8 (keratin) for ambient samples, and from 23.1 (vitellogenin) to 250 (keratin) for heat shock samples.

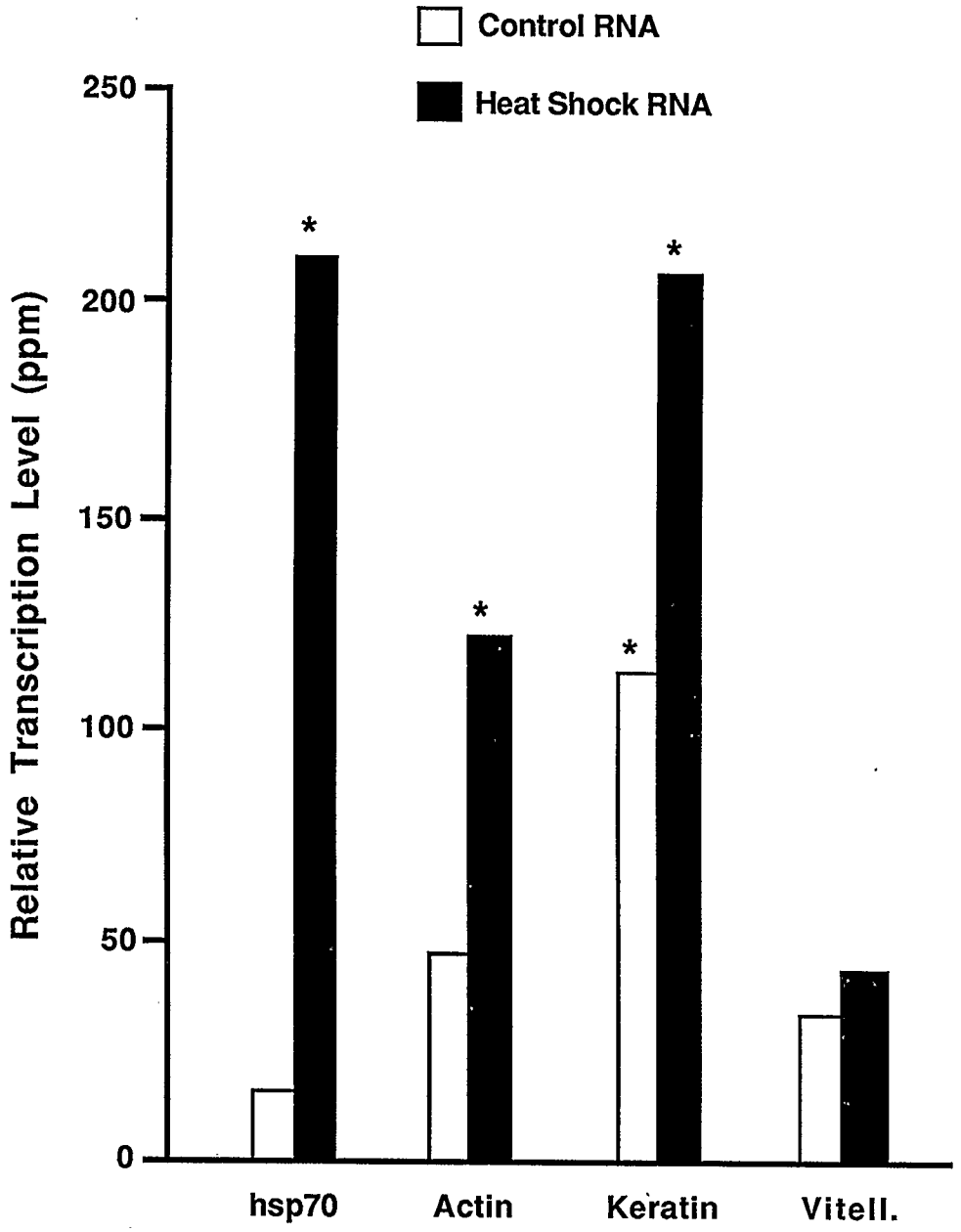


Figure 13. Relative transcription levels of the hsp70, actin, keratin, and vitellogenin genes in erythrocyte nuclei under ambient and heat shock conditions. As in Figure 11, data represent calculated means. Asterisks (*) indicate transcription that is significant at the 0.05 level compared to vitellogenin gene transcription.

compared to the ribosomal genes but are still significant. This result suggests that genes other than ribosomal may also be transcribed constitutively in erythrocytes. The keratin gene also shows increased transcription under heat shock conditions. Transcription of the keratin gene was induced more than two-fold over ambient levels.

To confirm that the results for the hsp70 gene were due to actual transcription of the gene and not simply an artefact, transcription of the hsp70 gene was assayed in erythrocytes treated with α-amanitin (Figure 14). As a control for this experiment, transcription of the ribosomal genes in the presence of α-amanitin was also examined.The concentration of α-amanitin used in this experiment has been shown to completely inhibit runoff transcription of genes transcribed by RNA polymerase II, while leaving runoff transcription of ribosomal genes unaffected (Williams *et al.*, 1979).

In cells not treated with α-amanitin, a heat shock-induced increase in hsp70 transcription was observed as before. Addition of α-amanitin to heat-shocked nuclei prior to runoff transcription prevented this increase, resulting in hybridization at the same levels as ambient samples - levels that were not significantly greater at the 0.05 level than the hybridization levels of the vitellogenin gene. Treatment of ambient nuclei with the drug did not significantly affect the amount of hybridization to the hsp70 gene. This confirms our assumption that cpm from ambient samples hybridizing to the hsp70 gene are spurious and not the result of true constitutive transcription of the hsp70 gene. The sensitivity of heat-induced hsp70 transcription to α-amanitin suggests that this is a real transcriptional event involving RNA polymerase II.

Treatment with α-amanitin caused an increase in cpm hybridized to the ribosomal genes over the untreated sample. This probably does not reflect an increase in the absolute

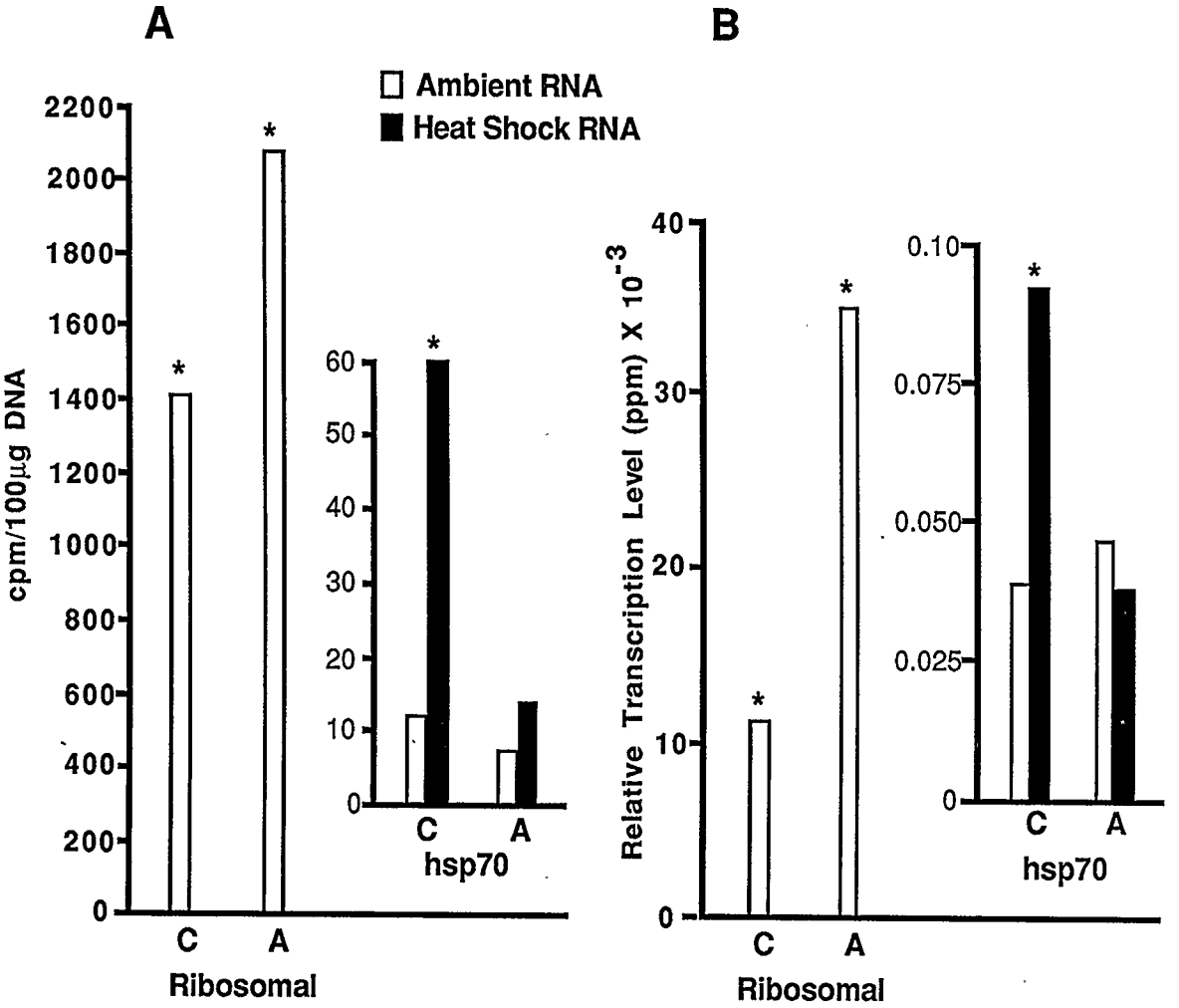


Figure 14. Transcription levels of the ribosomal genes under ambient conditions and (inset) the hsp70 gene under ambient and heat shock conditions, in the absence (C) or presence (A) of 5μg/ml α-amanitin. A, Absolute transcription levels; B, Relative transcription levels. Asterisks (*) indicate significant transcription. Actual cpm above background for this experiment ranged from 6.3 for hsp70-ambient-α-amanitin to 276.8 for ribosomal-α-amanitin.

transcription of the ribosomal genes, however. If labeled UTP is a limiting factor in the transcription reaction, inhibition of transcription of RNA polymerase II genes would result in an increase in the pool of radioactive UTP available for incorporation into ribosomal RNA, thus increasing the specific activity of the rRNA. It has not been conclusively determined, however, if such is the case. The dramatic increase in relative transcription of the ribosomal genes in α-amanitin-treated nuclei over control nuclei is further evidence that ribosomal transcription is unaffected by the drug, whereas transcription of polymerase II genes is greatly inhibited.

It was shown in Figure 1 that synthesis of hsp70 is maximal at 33°C and not detectable at 37°C. Transcription of the hsp70 gene at these temperatures was compared to determine if this temperature effect has a transcriptional basis (Figure 15). Hsp70 gene transcription was found to decline from high levels at 33°C to approximately ambient levels at 37°C. Transcription of the hsp70 gene is therefore not induced at the higher heat shock temperature, suggesting that the narrow temperature range that induces hsp70 synthesis is due to extreme sensitivity of hsp70 gene transcription to temperature.

C. Changes in Gene Expression During Erythropoiesis

Changes in transcription levels of the ribosomal, globin, actin, and hsp70 genes during differentiation of erythroid cells were examined. In these experiments, polychromatophilic-enriched samples consistently showed three- to ten-fold higher incorporation of ^{32}P-UTP into runoff transcripts than the other cell types, indicating that transcription reaches a peak in the polychromatophilic cells. As with the erythrocyte transcription studies, within each erythroblast cell type, heat-shocked nuclei showed higher incorporation of the labeled precursor into RNA than did ambient nuclei.

For these experiments, transcription attributable to contaminating cells was calculated

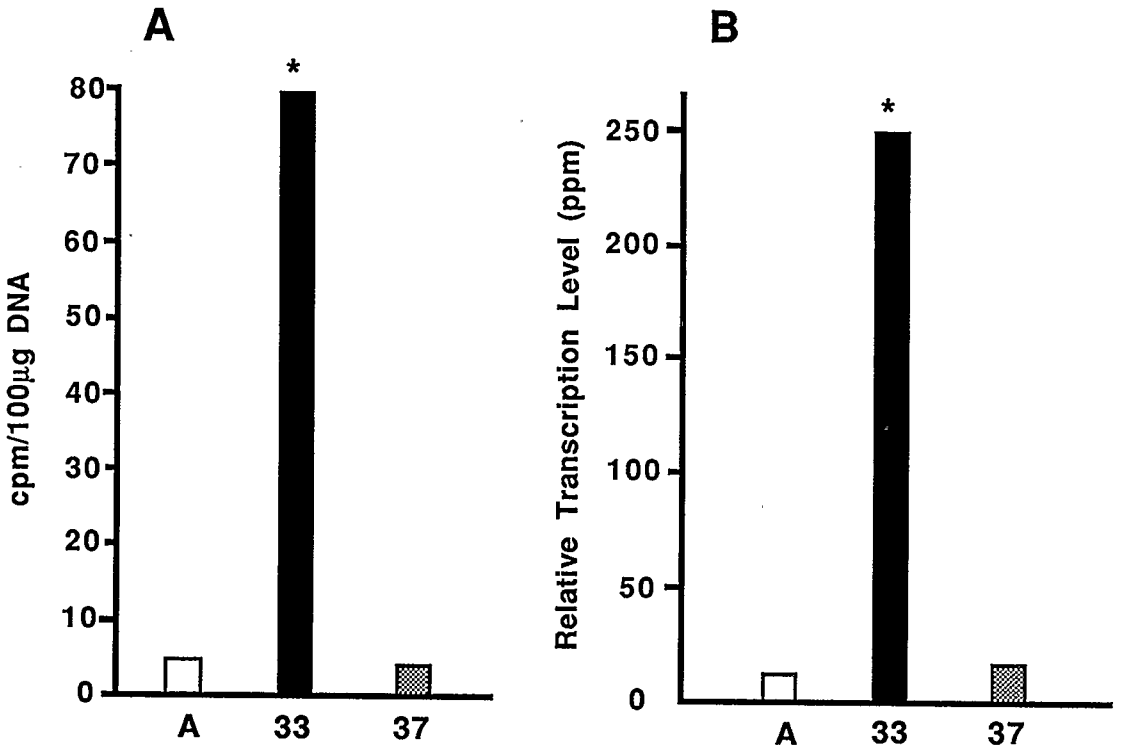


Figure 15. Transcription levels of the hsp70 gene at ambient temperature (A) and at heat shock temperatures of 33°C and 37°C. A, Absolute transcription levels; B, Relative transcription levels. Asterisks (*) indicate significant transcription. Actual cpm above background for this experiment ranged from 2.8 for 37°C to 60.1 for 33°C.

Results of the constitutive transcription assays for the ribosomal and globin genes are shown in Figures 16 and 17. Constitutive ribosomal gene transcription increases greatly from the basophilic to the polychromatophilic stage, decreases in orthochromatic erythroblasts, and finally increases again in erythrocytes (Figure 16). Because of the high amount of total transcription in the polychromatophilic erythroblasts, as mentioned previously, the difference in relative ribosomal transcription between these cells and basophilic erythroblasts is not as pronounced as the difference in absolute transcription (Figure 17).

Transcription of the ß-globin gene is maximal in polychromatophilic erythroblasts, the stage at which globin synthesis is first detected. Again, because of the high total transcriptional levels in these cells, ß-globin transcription makes up a lower proportion of the total transcription than in basophilic erythroblasts or orthochromatic erythroblasts. Absolute transcription of the gene decreases after the polychromatophilic stage and is completely repressed in the erythrocyte.

Changes in constitutive and inducible hsp70 gene transcription are presented in Figures 18 and 19. Transcription of the hsp70 gene is induced by heat shock at all stages of erythroid differentiation. The degree of induction, however, varies according to cell type. Basophilic erythroblasts and polychromatophilic erythroblasts show the highest levels of actual transcription of the hsp70 gene during heat shock. However, because the polychromatophilic erythroblasts are so active in total transcription, relative transcription of the gene in these cells is only slightly higher than that seen in orthochromatic erythroblasts. Relative transcription of the hsp70 gene is highest in in basophilic erythroblasts, suggesting that committment of the cells to the heat shock response is greatest at this earliest erythroblast stage and declines progressively as the cells mature. This parallels what was seen in the

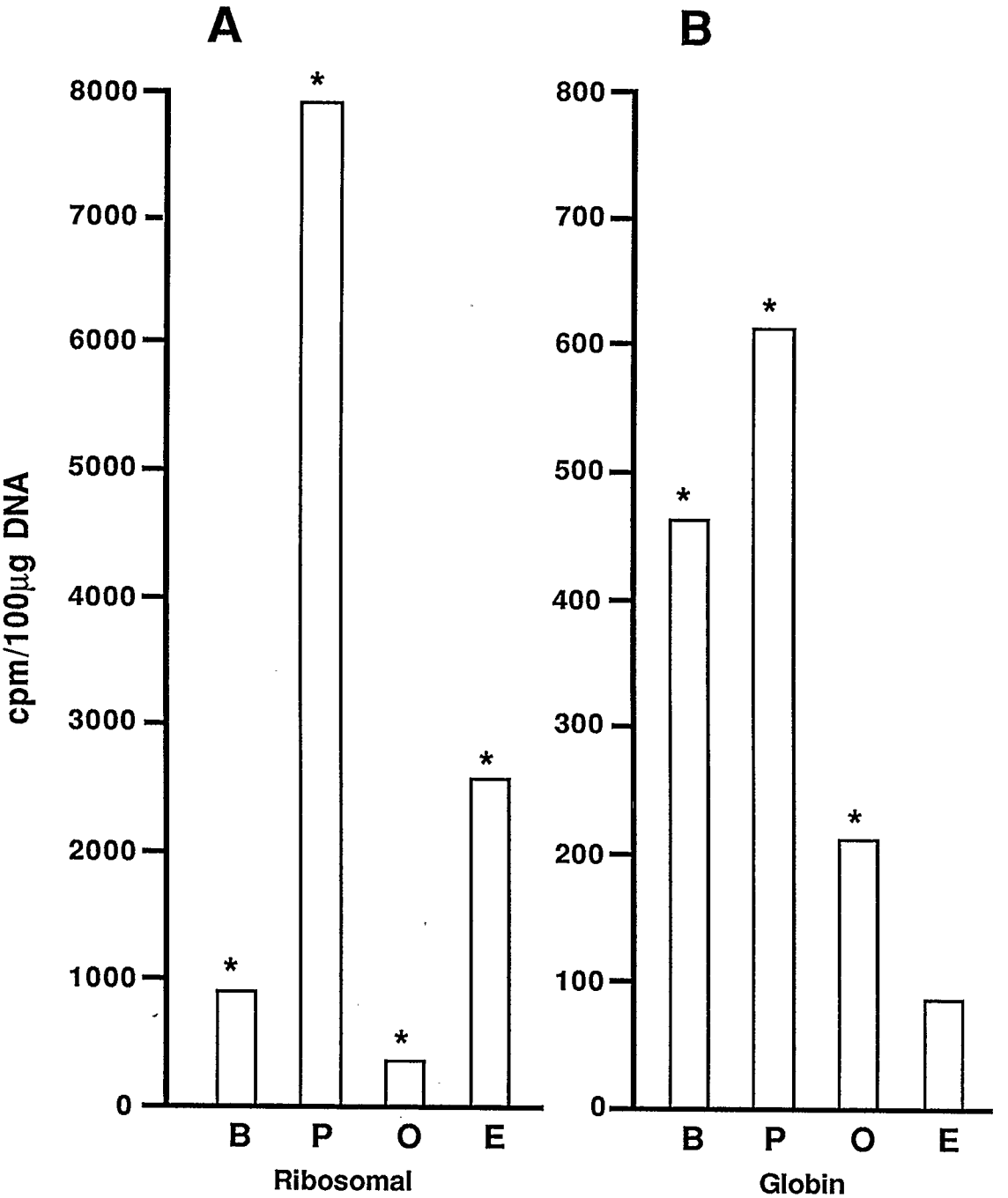


Figure 16. Absolute levels of constitutive transcription of the ribosomal and ß-globin genes during erythropoiesis. A, Transcription of the ribosomal genes; B, Transcription of the ß-globin gene. Erythroid cell types are represented as follows: Basophilic erythroblasts (B), polychromatophilic erythroblasts (P), orthochromatic erythroblasts (O), and erythrocytes (E). Asterisks (*) indicate significant transcription. Actual cpm above background ranged from 58.2 for erythrocyte globin to 299 for polychromatophilic ribosomal.

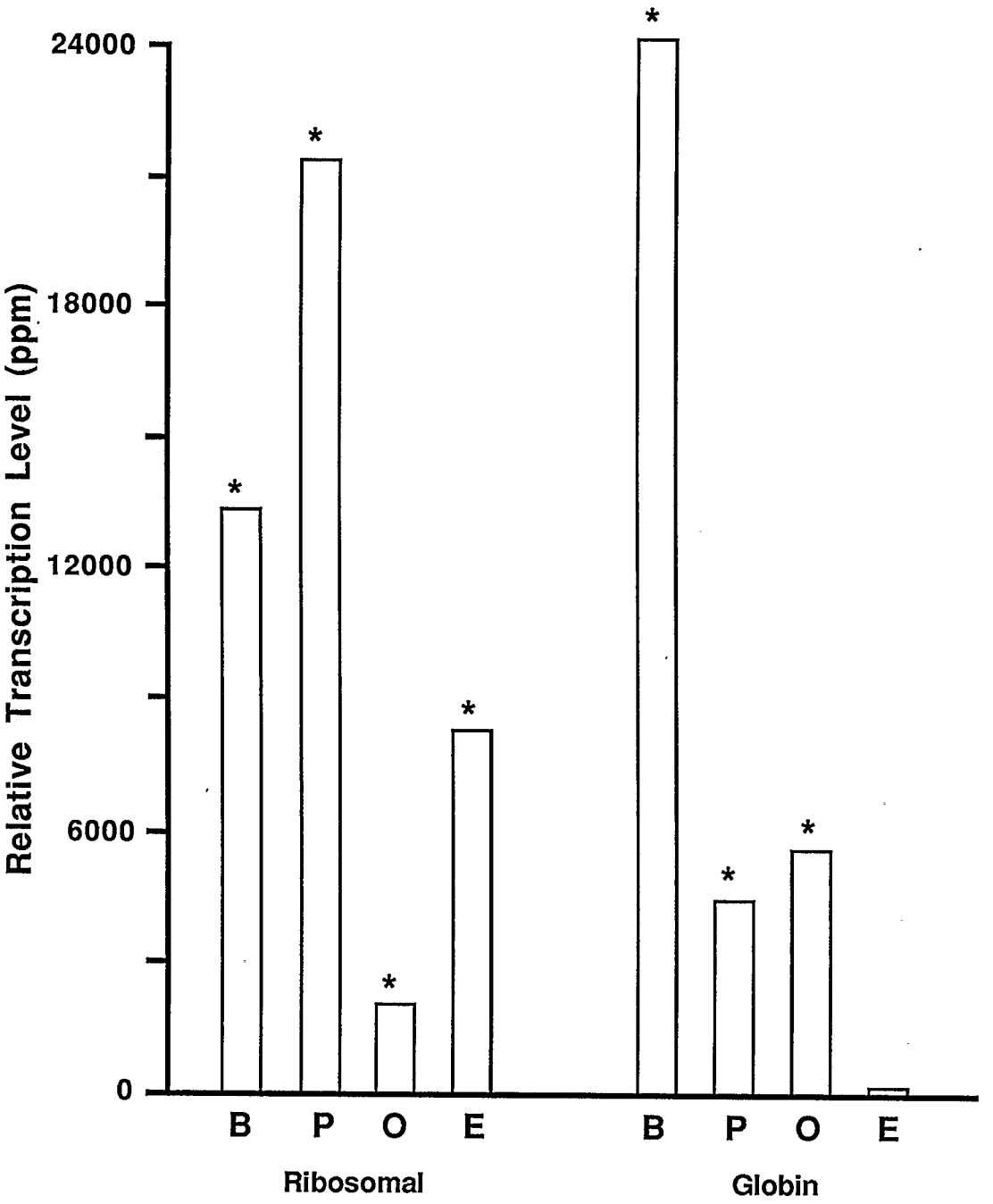


Figure 17. Relative transcription levels of the ribosomal and ß-globin genes during erythropoiesis, based on the absolute transcription levels presented in Figure 16. As in Figure 16, * indicates significant transcription.

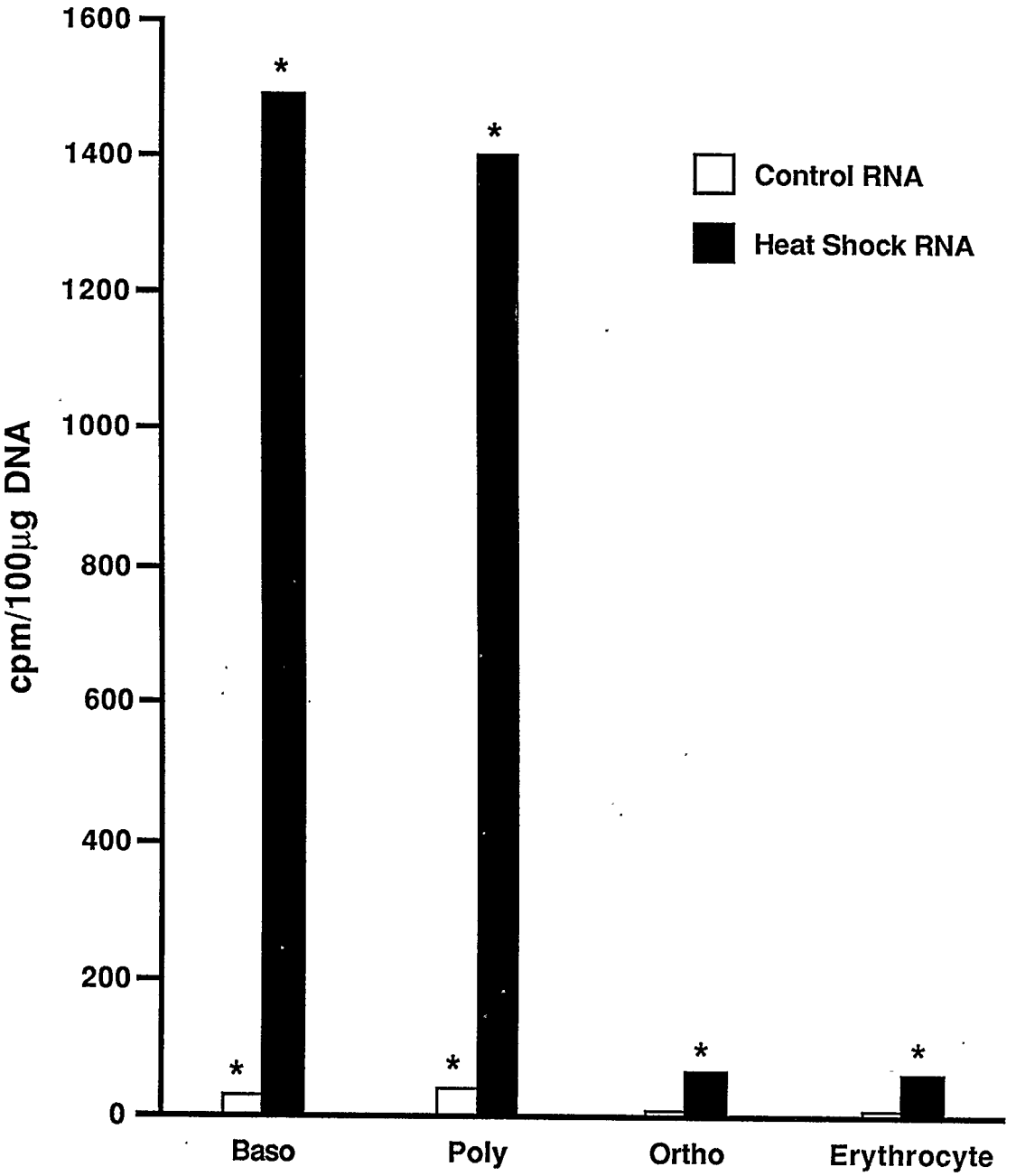


Figure 18. Absolute transcription levels of the hsp70 gene under both ambient and heat shock conditions during erythropoiesis. Erythroid cell populations are represented as follows: Basophilic erythroblasts (Baso), polychromatophilic erythroblasts (Poly), orthochromatic erythroblasts (Ortho), and erythrocytes. Asterisks (*) indicate significant transcription. Actual cpm above background ranged from 19.3 (erythrocyte) to 69.3 (poly) for ambient samples and from 93.9 (erythrocyte) to 338.5 (poly) for heat shock samples.

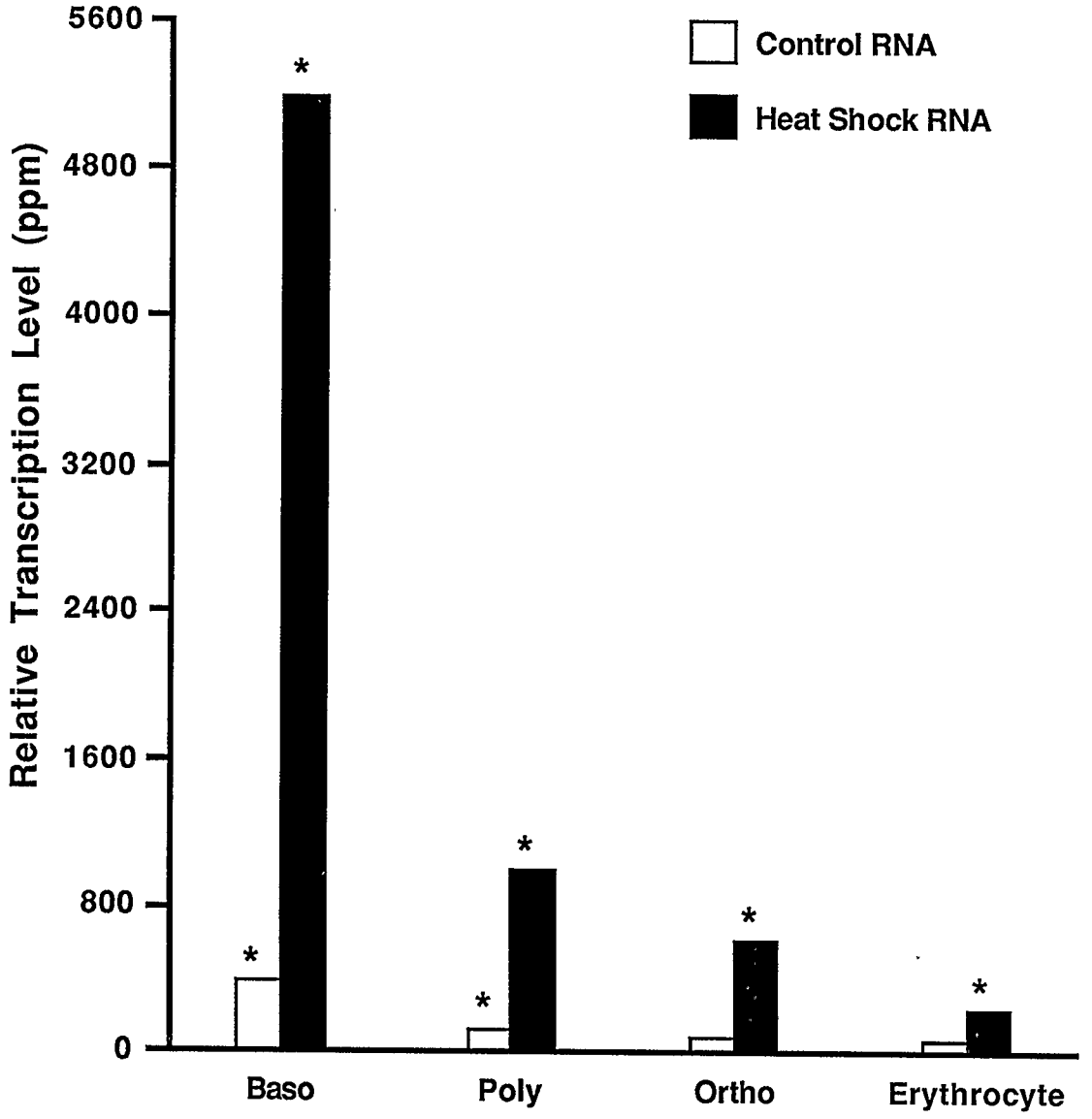


Figure 19. Relative transcription levels of the hsp70 gene under both ambient and heat shock conditions during erythropoiesis, based on the absolute transcription levels presented in Figure 18. As in Figure 18, * indicates significant transcription.

studies of protein synthesis in heat-shocked erythroblasts.

Transcription of an hsp70 gene was also seen in the basophilic and polychromatophilic erythroblasts at ambient temperature. Although accumulation of the hsp70 mRNA was not examined, there is no apparent constitutive synthesis of hsp70 in either of these stages, so this transcription is presumably for the storage of mRNA to be used in the constitutive synthesis of the hsp70-like protein in orthochromatic erythroblasts. It cannot be determined from the transcription assay whether the gene being transcribed is the hsp70 gene itself or an hsp70 cognate gene with sufficient sequence homology to cross-hybridize to the cloned hsp70 gene probe.

Constitutive transcription of actin genes decreases during differentiation, except that erythrocytes appear to show an increase in transcription of the gene over orthochromatic erythroblasts (Figures 20 and 21). The level of absolute transcription of actin genes (measured in cpm) in both of these cell types is not significant, however, so this apparent increase is probably spurious. Constitutive transcription of actin genes, then, decreases progressively, and is completely repressed by the orthochromatic stage.

Actin gene transcription is increased significantly by heat shock in polychromatophilic erythroblasts as well as erythrocytes. The heat-induction in polychromatophilic erythroblasts is much greater than in erythrocytes, again reflecting the repression of inducible synthesis that occurs between these stages.

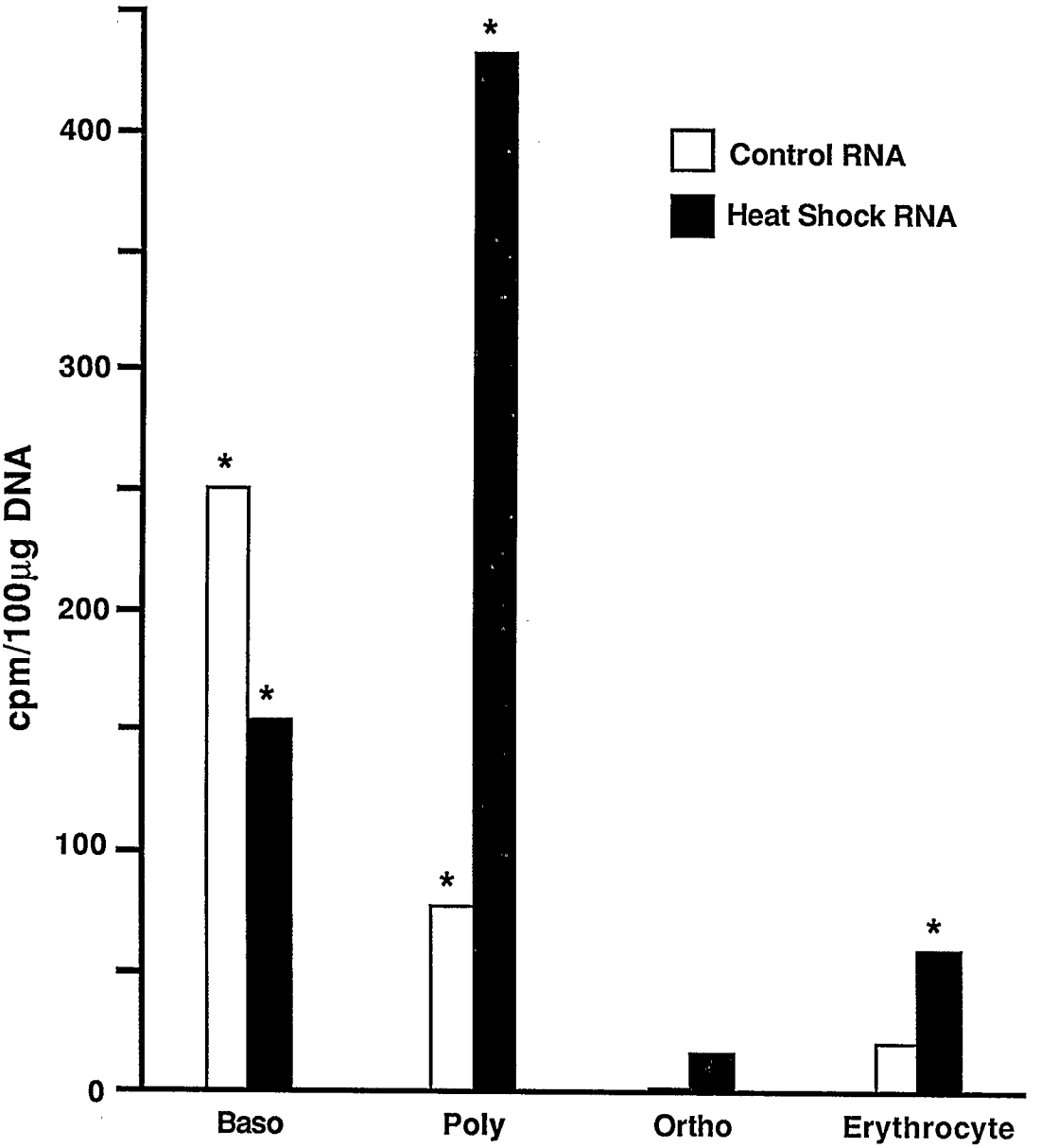


Figure 20. Absolute transcription levels of actin genes under both ambient and heat shock conditions during erythropoiesis. Erythroid cell populations are represented as in Figure 18. Asterisks (*) indicate significant transcription. Actual cpm above background for this experiment ranged from 5.1 (ortho) to 75.9 (baso) for ambient samples and from 22.3 (ortho) to 175 (poly) for heat shock samples.

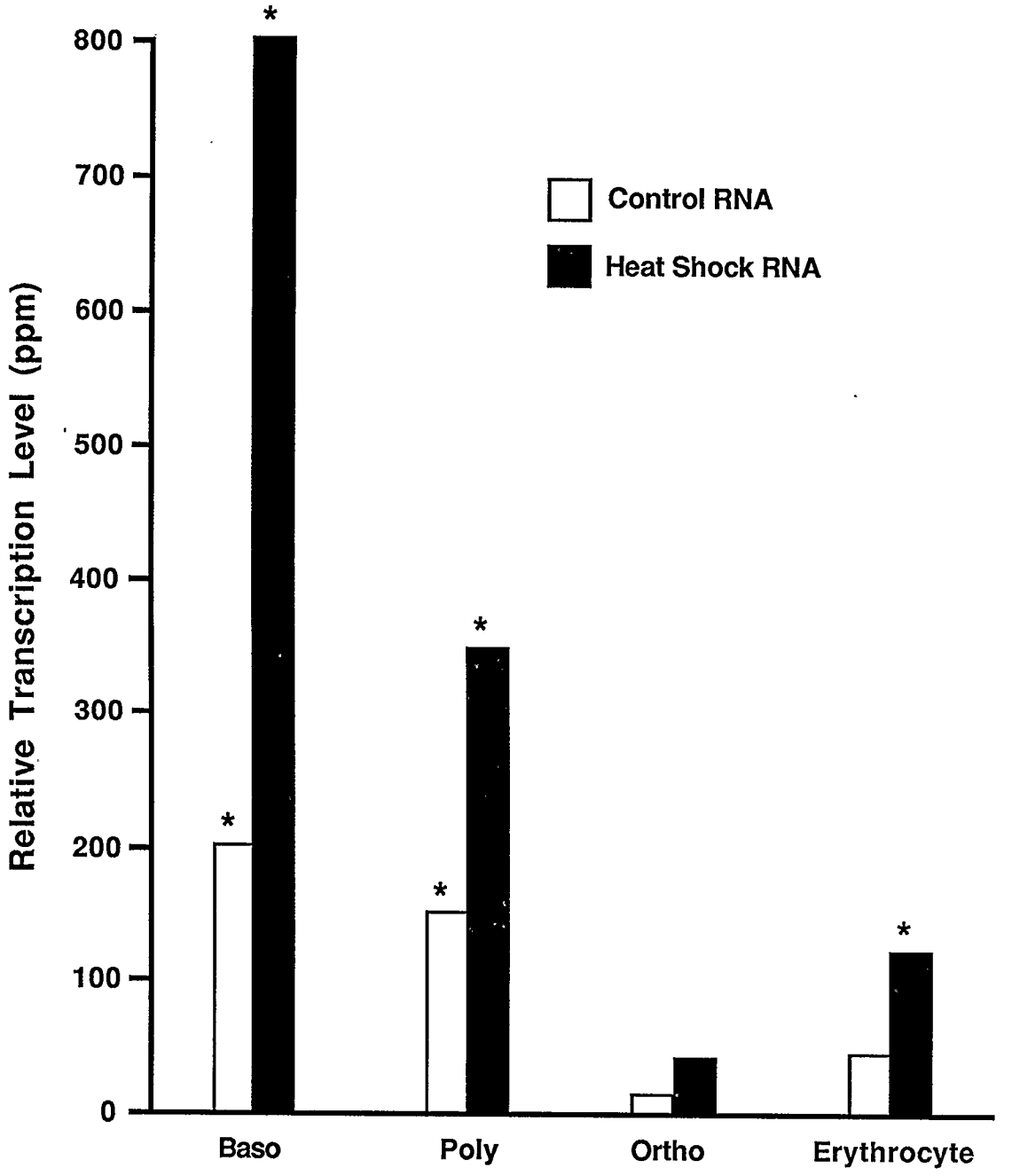


Figure 21. Relative levels of actin genes under both ambient and heat shock conditions during erythropoiesis, based on the absolute transcription levels presented in Figure 20. As in Figure 20, * indicates significant transcription.

DISCUSSION

This study demonstrates that *Xenopus laevis* erythrocytes are not as synthetically repressed as has been previously reported. Prior studies of *Xenopus* erythrocytes have examined only constitutive synthesis of protein (Maclean *et al.*, 1969) and RNA (Maclean *et al.*, 1973; Hentschel and Tata, 1978) using less refined techniques than are presently available. The present study examines both constitutive and inducible gene expression throughout erythropoiesis. This analysis was conducted at both the transcriptional and translational levels. Notwithstanding the reduction of RNA and protein synthesis that does occur during erythropoiesis, erythrocytes are capable of transcription of specific constitutive and inducible genes and of both constitutive and inducible protein synthesis.

The changes during differentiation in the synthesis of specific proteins were assessed by analyzing the potential for heat shock protein synthesis at the various erythroid developmental stages. Results show that the pattern of hsp synthesis becomes less complex as erythroid cells mature. However, in spite of the progressive restriction of the erythroid heat shock response, terminally-differentiated erythrocytes retain the ability to respond to elevated temperatures by synthesizing one heat shock protein, hsp70. The range of temperatures (30°C to 33°C) that induce hsp70 synthesis is quite narrow as compared to *Xenopus* embryos, which synthesize hsps at temperatures up to 37°C (Heikkila *et al.*, 1985). Although the reason for the narrow temperature range in erythrocytes is not known, one possible explanation is that physiological responses by the animals to elevated temperatures may prevent erythrocytes in the circulation from being exposed to temperatures above 33°C, so that a heat shock response induced by higher temperatures would not normally be required in erythrocytes.

In addition to heat shock-induction of hsp synthesis, there appears to be developmental

regulation of the synthesis of heat shock-related proteins. An hsp70-like protein and an hsp87-like protein appear to be synthesized constitutively in orthochromatic erythroblasts. Constitutive synthesis of hsp-like proteins may be important in a variety of normal developmental processes. Heat shock genes are constitutively expressed during oogenesis (Zimmerman *et al.*, 1983) and embryogenesis (Sirotkin and Davidson, 1982) of *Drosophila*, during early mouse embryogenesis (Bensaude and Morange, 1983), and after fertilization of *Xenopus* eggs (Browder *et al.*, 1985).In addition to these examples involving early developmental events, synthesis of hsp70-like proteins has been correlated with cell differentiation in a number of systems. Synthesis of an hsp70-related protein is induced coincidentally with the induction of differentiation of a human erythroid cell line by hemin (Singh and Yu, 1984). In addition, a shift in temperature from 25°C to 37°C induces differentiation events in the parasitic protozoan *Leishmania* (Lawrence and Robert-Gero, 1985; Hunter *et al.*, 1984) and the fungus *Histoplasma capsulatum* (Lambowitz *et al.*, 1983) as well as inducing the synthesis of heat shock proteins. Although none of these studies report a direct relationship between hsp synthesis and differentiation, all suggest that hsps or hsp-related proteins may be involved in the differentiation process.

Developmental regulation in addition to inducible regulation of hsp-like synthesis suggests the possibility of multiple regulatory sites for the hsp70 gene, as has been reported for the hsp26 gene in *Drosophila* (Cohen and Meselson, 1985). Examination of the regulatory elements of the *Xenopus* hsp70 gene may prove useful in the study of multiple gene regulatory sites in vertebrates. *Xenopus* erythroid cells may also provide an excellent system for the study of control of translation of heat shock mRNAs. As discussed previously, constitutive synthesis of the hsp70-like protein in orthochromatic erythroblasts appears to use transcripts synthesized in basophilic and polychromatophilic erythroblasts,

although this has not been verified by studying accumulation of hsp70 mRNA. No constitutive synthesis of an hsp70-like protein has been detected at the basophilic or polychromatophilic stages. Thus, there is likely a stage-specific mechanism for utilization of the hsp70 mRNA for protein synthesis.

Another unusual aspect of developmental regulation of hsp synthesis was observed in orthochromatic erythroblasts. Whereas the heat shock response in basophilic erythroblasts involves the synthesis of most of the *Xenopus* hsps, and later stages synthesize fewer hsps, synthesis of an additional protein - hsp65 - is induced during erythropoiesis only in heat-shocked orthochromatic erythroblasts. As mentioned previously, heat shock-inducible synthesis of hsp65 has also been detected in artificially activated eggs (Pollock and Browder, unpublished results) and neurulae (Nickells and Browder, unpublished results) of *Xenopus*. Because it only appears to be synthesized at certain developmental stages, hsp65 may have a specialized function in the heat shock response of specific cell types.

In vitro nuclear runoff transcription was used to assay the transcription of various genes in erythroid cells. This technique is extremely useful because it allows direct measurement of the level of transcription of a specific gene occurring at a given point in time. Techniques such as Northern blot analysis and S1 protection only allow inference of gene activity based on accumulation of specific RNA sequences. Such inferences are limited, because changes in transcriptional activity and messenger stability are indistinguishable by this technique. Runoff transcription, however, addresses only the question of transription level and does not allow examination of accumulation of specific RNA sequences. This limits conclusions that may be drawn regarding utilization of mRNA molecules for protein synthesis.

Transcription of the hsp70 gene during heat shock in erythrocytes was assayed in

order to determine if the erythrocyte heat shock response is regulated primarily at the transcriptional level or only at the level of translation. The conclusions of Maclean *et al.* (1973) that *Xenopus* erythrocytes are transcriptionally inert would suggest that the latter possibility is correct. However, results presented herein, in which hsp70 synthesis in erythrocytes was shown to be sensitive to actinomycin D, would argue for the former.The nuclear runoff transcription assay reveals that transcription of the hsp70 gene is greatly elevated in erythrocytes during heat shock. The transcription level of the hsp70 gene in heat-shocked erythrocytes is lowered by treatment with α-amanitin to the ambient level, which is not significantly higher than spurious levels. These results strongly suggest that the heat shock response in *Xenopus* erythrocytes is regulated by transcription involving RNA polymerase II activity. Transcriptional regulation of the heat shock response has not been conclusively demonstrated, however. Northern blot analysis or S1 protection assays are required to determine if the newly-synthesized hsp70 transcripts accumulate during heat shock. Otherwise it cannot be concluded that this RNA is translated. However, other supporting evidence for transcriptional regulation exists. Transcription inhibitors, as described above, inhibit both transcription of the hsp70 gene and synthesis of hsp70, indicating that the two events are related. The coordinate increase in hsp70 gene transcription and hsp70 synthesis observed in this study is a different situation from that seen in chicken erythroid cells. As discussed in the Introduction, reticulocytes from chicken require transcription for the heat shock response, but transcription of the hsp70 gene itself is not increased during heat shock.

Although primary regulation of heat shock-induction of hsp70 synthesis in *Xenopus* erythrocytes appears to occur at the transcriptional level, translational regulation may be involved in the synthesis of certain other proteins. Upon heat shock, the synthesis of some

proteins is repressed, whereas the synthesis of other proteins (those proteins classified as being heat shock-tolerant) is not. This suggests that the protein synthetic machinery may be able to distinguish between different mRNA species. (Constitutive protein synthesis in these cells requires no transcription.) How selective transcript utilization might be accomplished is unknown, but it may involve recognition of signals in the 5' untranslated leader of mRNA molecules, as has been proposed for *Drosophila* hsp70 (McGarry and Lindquist, 1985). The situation in *Xenopus* erythrocytes is more complex than in *Drosophila*, however, involving heat-tolerant as well as heat shock proteins, so more than one type of signal would be required.

Transcriptional activity in erythrocytes was found to be more extensive than inducible transcription of the hsp70 gene. The ribosomal genes were observed to be constitutively transcribed at high levels in erythrocytes. Ribosomal transcription was previously reported in up to 12% of erythrocytes (Maclean *et al.*, 1973), but the high levels reported here may be indicative of more widespread ribosomal transcription in erythrocytes. However, *in situ* hybridization of a labeled ribosomal gene probe to RNA in fixed erythrocytes is required to demonstrate this. Constitutive transcription other than ribosomal was also observed. Significant amounts of labeled RNA from nuclei of ambient cells hybridized to the epidermal keratin gene. This was contrary to expectation, as this gene has been reported to be expressed only in epidermal cells of *Xenopus* embryos (Jonas *et al.*, 1985). The keratin gene is a member of the intermediate filament (IF) protein gene family, a family of genes sharing sequence homology within a large central domain (reviewed by Steinert *et al.*, 1985). However, even closely related cytokeratin genes will not cross-hybridize to the epidermal keratin gene at the stringency used in this study (T. Sargent, personal communication), so the transcription observed is likely due to the epidermal keratin gene itself.

Transcription of the keratin gene in heat-shocked erythrocytes was greater than in ambient cells, suggesting that this gene may act as a heat shock gene. Such a possibility has precedent, as it has been proposed recently that the small hsps of *Drosophila* are IF proteins (Leicht *et al.*, 1986). However, possible translation of keratin mRNA during heat shock was not investigated, so no conclusions can be drawn regarding the role of keratin transcription in the heat shock response.

Transcription of actin genes is also induced during heat shock. This result and the accumulation of actin mRNA in *Xenopus* embryos during heat shock (Heikkila *et al.*, 1985) suggest that actin genes may act as heat shock genes in *Xenopus*. Such a suggestion is interesting, considering that one of the heat shock-tolerant proteins seen in erythrocytes has a M_r of 43kD, which is approximately the M_r of actin. A relationship between actin and the 43kD heat shock-tolerant protein has not been established, however. Also intriguing is the recent report that the heat shock response in *Drosophila* can be induced by the accumulation of abnormal (truncated) actin proteins (Karlik *et al.*, 1984). Abnormal (heat-denatured) actin may therefore act as a trigger molecule for the heat shock response. If the abnormal actin is then degraded, normal feedback mechanisms governing actin synthesis might induce actin synthesis to restore levels of the protein. Thus, transcription of actin genes would be induced indirectly as a result of heat shock, and not directly by the heat shock itself. With such a mechanism, it would be expected that higher temperatures would create more denatured actin than heat shock at lower temperatures, resulting ultimately in higher levels of actin synthesis.Synthesis of the putative actin protein follows such a pattern - the protein appears to be synthesized at higher levels in erythrocytes at 37°C than at lower heat shock temperatures.

It might be argued that the heat-induced increases in transcription of the hsp70, actin,

and keratin genes do not represent a true heat shock response as such, but are merely the reflection of an increased metabolic rate in the cells due to increased temperature. Metabolic rates increase with temperature; the ratio of metabolic rates at temperatures separated by 10 C^{o} is called a Q_{10} value. The Q_{10} value for a temperature increase from 25°C to 35°C has been determined in the cricket frog to be approximately 2.9 (Dunlap, 1971). Such a Q_{10} effect might explain the heat-induced transcription observed: RNA polymerase might be more active at 33°C than at 23°C, resulting in increased transcription that might appear as induced transcription of certain genes. However, this possibility is very unlikely for a number of reasons. Radioactive cpm hybridizing to the hsp70 gene showed a ten-fold increase over ambient levels during heat shock. Such an increase is much greater than can be explained by a temperature-mediated metabolic increase. In addition, heat shock-induced increases in transcription of the hsp70, actin, and keratin genes were all reflected in the relative transcription level as well as in the absolute transcription level. If the apparent increases in transcription of these genes were due to an increase in RNA polymerase activity, all transcription would be expected to increase to a similar extent. The level of transcription of a single gene would therefore not change relative to total transcription.

Ribosomal transcription has been determined to comprise 54% of all runoff transcription in isolated *Xenopus* kidney cell nuclei (Reeder and Roeder, 1972). Surprisingly, constitutive ribosomal transcription in erythrocytes accounts for less than 1% of the total incorporation of labeled UTP into RNA. As discussed in the Introduction, Hentschel and Tata (1978) reported that RNA polymerase I is absent from *Xenopus* erythrocytes. The high levels of α-amanitin-resistant absolute ribosomal transcription demonstrated here clearly indicate that this is not so. However, the percentage of total UTP

incorporation comprised of ribosomal transcription demonstrates that RNA polymerase I activity is lower than expected, based on the results for kidney cells. This raises a question regarding the nature of the remaining molecules into which the UTP is incorporated. One possibility is that spurious transcription accounts for most of the remaining incorporation. However, based on the values of spuriously-incorporated radioactivity hybridizing to the 1.5 kb vitellogenin cDNA clone, and assuming (1) a total genome size for *Xenopus* of 6.2×10^6 kb (Davidson *et al.*, 1973), (2) that spurious transcription consists of random, non-regulated transcription events, and (3) that the vitellogenin gene is representative of the nonribosomal fraction of the genome, calculations reveal that spurious transcription would result in substantially more incorporation of the labeled precursor than was observed in most experiments. Clearly, not all of the genome is involved in spurious transcription; some sequences must be refractory to spurious transcription. The observation that incorporation of labeled UTP into hsp70 transcripts under ambient conditions is resistant to α-amanitin implies that the spurious incorporation may not involve a transcriptional event at all. We must also entertain the possibility that legitimate transcription of specific unidentified genes occurs.

When the spurious levels of transcription of the vitellogenin gene are compared to the transcription levels of the ribosomal genes, an anomaly becomes apparent. If all of the 940 ribosomal genes (each gene having a length of 11.75 kb - Bakken *et al.*, 1982) in the diploid *Xenopus* genome (Brown and Weber, 1968) were transcribed at the same level as the four copies of the vitellogenin sequence (which has a length of 1.5 kb - Wahli *et al.*, 1977), incorporation of ^{32}P-UTP into rRNA would be more than five times as great as was detected. Obviously, not all of the ribosomal genes are being transcribed in erythrocytes. This is not without precedent, though; for example, only 1% of the ribosomal genes are actively transcribed in *Xenopus* embryos (Brown and Littna, 1966).

Examination of transcription levels of the ribosomal, globin, hsp70, and actin genes at different stages during erythropoiesis reveals that, in general, transcription of these genes decreases gradually over the course of differentiation. There is an exception to this pattern, however. Transcription of the ribosomal genes increases early in erythropoiesis, then decreases, but increases again in erythrocytes over the level in orthochromatic erythroblasts. It is not known why this would occur.

The relative levels of hsp70 gene transcription under both ambient and heat shock conditions follow the general pattern of a decrease in transcription over the course of differentiation. Constitutive transcription of the gene occurs early in the erythropoietic pathway; as mentioned previously, these transcripts appear not to be translated but may be stored for later use. Transcription of the hsp70 gene is greatly induced by heat shock at all stages of erythroid differentiation. However, the amount of the heat shock-induced increase in transcription declines during differentiation, reflecting the general synthetic repression.

In conclusion, RNA and protein synthetic activity appear to become reduced during erythropoiesis in *Xenopus laevis*. The synthesis of specific proteins, as reflected in the heat shock-induced synthesis of heat shock proteins, becomes restricted, and both constitutive and inducible transcription decrease over the course of differentiation. However, in spite of this repression, terminally-differentiated erythrocytes are capable of inducible synthesis. These cells respond to heat shock by synthesizing hsp70, and this synthesis is paralleled by transcription of the hsp70 gene. Heat shock-inducible transcription is also seen for the actin and keratin genes. In addition to inducible transcription, constitutive transcription was also seen in erythrocytes. The ribosomal genes and the keratin gene are transcribed under normal circumstances in these cells. These results suggest that the *Xenopus* erythrocyte nucleus, which has been thought to be more metabolically inert than any other, still retains significant

actual and potential synthetic activity. This implies that the genetic repression that accompanies erythroid differentiation may not be as consummate as has been believed.

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