REVIEW ARTICLE

Xenopus transcription factors: key molecules in the developmental regulation of differential gene expression

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INTRODUCTION

Xenopus laevis, the clawed frog, has many advantages for research concerning the regulation of differential gene expression during development. The large size and accessibility of the *Xenopus* egg facilitates physical manipulations such as tissue transplantation and microinjection. Consequently we have a very complete picture of the various events necessary for embryogenesis to be successfully completed. A biochemical approach to embryology has also met with much success. Inspired by the results of microinjection experiments in which *Xenopus* eggs and oocytes were used as living test-tubes (reviewed by Gurdon & Melton, 1981), investigators have established cell-free systems derived from oocytes, eggs and embryos that clearly reproduce regulatory events defined *in vivo*. In this Review I will focus primarily on the molecular mechanisms regulating the differential transcription of genes at the level of protein–DNA interactions. For a comprehensive review of the role of growth factors and signal transduction in *Xenopus* embryogenesis see Dawid *et al.* (1990). *In vitro* transcription by all three classes of eukaryotic RNA polymerase has been achieved using *Xenopus* egg or oocyte extracts. Many of the molecules required to mediate the transcription process have been fractionated and in some cases purified. Many other transcription factors have been cloned by homology (see Table 1). The combination of our capacity to manipulate the amphibian embryo, knowledge of the processes influencing the fate of particular cells, cloned transcription factors and functional *in vitro* assays offers a unique opportunity to clarify the molecular mechanisms determining when and where a

particular gene will be expressed in a particular tissue in the developmental program. This review describes recent progress towards achieving this goal.

EUKARYOTIC TRANSCRIPTION: A BRIEF OVERVIEW

There are three classes of eukaryotic genes, which differ in their organization, in the proteins that regulate their transcription and in the RNA polymerase that transcribes the gene. Class I genes encode the 18 S and 28 S ribosomal RNA molecules. Class II genes encode the wide variety of mRNA molecules that will be translated into protein. Class III genes encode the small tRNA molecules involved in translation and the 5 S RNA molecule present in the ribosome. Each of these gene classes contains different *cis*-acting DNA sequences within its promoter (a combination of regulatory elements generally with a fixed orientation, close to the start of transcription) and enhancer (a combination of regulatory elements that are both orientation-independent and distant from the start of transcription). These different *cis*-acting DNA sequences interact with distinct proteins called transcription factors. The interaction of these specific proteins with promoters and enhancers represents the first stage in assembling a transcription complex. Other transcription factors that are not themselves sequence-specific DNA-binding proteins interact with the DNA-binding proteins present at the promoter or enhancer to create a multiprotein–DNA complex. This transcription complex differs on each of the three classes of eukaryotic gene and is recognized by a distinct RNA polymerase

Table 1. Listing of *Xenopus* transcription factors

Transcription factor	Biochemical purification	Cloning	Genes regulated	Gene class
TFIIIA	Engelke <i>et al.</i> (1980)	Ginsberg <i>et al.</i> (1984)	5S RNA genes	Class III
TFIS (x UBF)	Dunaway (1989), Pikaard <i>et al.</i> (1989)	Not done	28 S and 18 S rRNA	Class I
Y-box factors	Not done	Tafari & Wolffe (1990)	hsp 70	Class II
Oestrogen receptor	Knowland <i>et al.</i> (1984)	Weiler <i>et al.</i> (1987)	Vitellogenin; oestrogen receptor	Class II
Thyroid hormone receptor	Not done	Yaoita & Brown (1990)	Thyroid hormone receptor	Class II
Homeodomain proteins				
Xhox-1	Not done	Carasco <i>et al.</i> (1984)	Unknown	Class II
Xhox-3		Harvey <i>et al.</i> (1986)		
Xhox-36		Condie & Harland (1987)		
MyoD	Not done	Hopwood <i>et al.</i> (1989), Scales <i>et al.</i> (1990)	Muscle specific	Class II
B1 (USF)	Kaulen <i>et al.</i> (1991)	Kaulen <i>et al.</i> (1991)	TFIIIA	Class II
KTF-1	Snape <i>et al.</i> (1990)	Not done	Epidermal keratin	Class II
OCT-1	Not done	Smith & Old (1990), Baltzinger <i>et al.</i> (1990)	Not known	Class II
ets1/ets2	Not done	Stiegler <i>et al.</i> (1990), Wolff <i>et al.</i> (1990)	Not known	Class II

Abbreviations used: ICR, internal control region; TF, transcription factor.

Fig. 1. The assembly of a transcription complex on the promoter of any eukaryotic gene

The site of transcription initiation (arrow) is immediately next to the promoter which contains binding sites a and b (1) for transcription factors A and B (2). Once the specific DNA-binding proteins A and B are bound to the promoter then non-DNA-binding transcription factors, such as C, can associate to form the complete transcription complex (3). RNA polymerase recognizes this complex and initiates transcription (4).

(I, II and III). The process of assembling a transcription complex and of transcribing a eukaryotic gene is shown in Fig. 1.

CLASS III GENES: RECONSTRUCTING DEVELOPMENTAL REGULATION *IN VITRO*

Xenopus embryos undergo rapid development in a hostile environment. To facilitate the developmental process, each egg has stores of ribosomes and other components of the translational apparatus that are over 200 000-fold in excess of those found in a normal somatic cell. This represents a huge requirement for ribosome synthesis during oogenesis (the developmental period of several months leading to a mature oocyte within the animal). The frog has used two evolutionary strategies to accomplish this high rate of synthesis of ribosomal components: the class I genes encoding 28 S and 18 S rRNA are amplified extrachromosomally and the class III genes encoding 5 S RNA are present in a large auxiliary multigene family that is transcribed only in oocytes.

The amplified 28 S and 18 S rRNA genes are lost during embryogenesis and the auxiliary 5 S RNA genes (oocyte-type) are repressed in somatic cells. A second, much smaller, family of 5 S RNA genes (somatic-type) remains active.

The abundant rRNA genes were the first to be isolated and cloned. Each repeated DNA sequence containing a 5 S RNA gene (120 bp) is less than a kilobase in length, facilitating manipulation using molecular genetic techniques. Injection of 5 S DNA into oocyte nuclei resulted in the synthesis of 5 S RNA. This *in vivo* assay was followed by the development of oocyte nuclear extracts in which mutants of 5 S DNA could be tested for transcriptional activity. The key regulatory elements for transcription were found within the 5 S RNA gene and comprised the so-called internal control region (ICR; Brown, 1982).

Roeder and colleagues had originally fractionated the three classes of RNA polymerases from *Xenopus* embryos (Roeder, 1974). RNA polymerase III would not accurately transcribe a 5 S RNA gene in isolation. Only after the genes were complexed with other proteins would RNA polymerase III be able to accurately initiate and terminate transcription (Parker & Roeder, 1977). Fractionation of oocyte extracts led to the purification of a protein that bound to the ICR (Engelke *et al.*, 1980). This protein (TFIIIA), together with two other protein fractions (TFIIIC and TFIIIB, isolated from oocyte extracts by chromatography on phosphocellulose) is required for transcription of 5 S S RNA genes by RNA polymerase III. Until recently TFIIIA was the only eukaryotic transcription factor available in pure form; a great deal of highly significant research has been done with this protein.

Transcription factor (TF) IIIA

Growing *Xenopus* oocytes store 5 S RNA in a ribonucleoprotein particle which contains a single molecule of 5 S RNA and a single molecule of TFIIIA. The abundance of this storage particle greatly facilitated the purification of large quantities of TFIIIA (Pelham & Brown, 1980; Honda & Roeder, 1980). TFIIIA binds to both the 5 S RNA gene and to the product of transcription, 5 S RNA; this suggests an auto-regulatory circuit that will be discussed later. The gene encoding TFIIIA was cloned and sequenced, revealing a repetitive structure within the protein (Ginsberg *et al.*, 1984; Taylor *et al.*, 1986; Miller *et al.*, 1985). This repeating unit of the protein has been called a zinc finger since each of the repeats might associate with a zinc atom (Hanas *et al.*, 1983; Miller *et al.*, 1985).

The structure of TFIIIA is now believed to consist of nine zinc fingers and a C-terminal domain which does not bind DNA. A single molecule of TFIIIA binds to each 5 S RNA gene. The zinc fingers are arrayed in a linear fashion along the ICR, such that the C-terminus of the protein projects towards the 5' end of the 5 S RNA gene (Smith *et al.*, 1984; Vrana *et al.*, 1988; Fig. 2a). The association of TFIIIA with the gene is necessary to initiate the binding of the other transcription factors required to assemble a transcription complex.

The transcription complex: the importance of stable protein-DNA interactions

As already described, a complete transcription complex on the 5 S RNA gene contains not only the specific DNA binding protein TFIIIA but also TFIIIB and TFIIIC. The incorporation of TFIIIC and TFIIIB into the transcription complex greatly extends protein-DNA interactions on the 5 S RNA gene (Fig. 2b), and alters those of TFIIIA with the ICR (Wolffe *et al.*, 1986; Wolffe & Morse, 1990). These specific protein-DNA contacts

Fig. 2. Transcription complex assembly on a *Xenopus* somatic 5 S RNA gene

(a) TFIIIA binds to the internal control region (hatched box) of the 5 S RNA gene (open arrow). The lengths of the various regions are indicated in base pairs relative to the site of transcription initiation (+1, arrow). TFIIIA consists of two domains, the N-terminal DNA-binding domain (red) and a C-terminal region involved in protein-protein contacts with other transcription factors (black). (b) Once TFIIIA is bound to the 5 S RNA gene, two other transcription factors associate (TFIIB and TFIIC) to assemble the complete transcription complex. Protein-DNA contacts in this complex (indicated in red above the gene) extend from -30 to +159. Almost 200 bp of DNA is organized into the transcription complex.

potentiate the efficiency and accuracy of the transcription process; however, they are not absolutely essential.

The stable association of multiple transcription factors with eukaryotic genes has been described *in vitro* and *in vivo*. The significance of such stable interactions is that, in many cells, stable patterns of gene activity are maintained for long periods of time and, in the case of a terminally differentiated cell, until cell death. However, for genes requiring transcriptional activity to be modulated, the transient association and dissociation of transcription factors is advantageous (Wolffe, 1990a). Transcription complexes assembled on somatic and oocyte 5 S RNA genes typify these two modes of interaction between transcription factors and promoter elements (Bogenhagen *et al.*, 1982; Wolffe & Brown, 1987; Darby *et al.*, 1988).

First, the stable association of transcription factors with a somatic 5 S RNA gene will be discussed. Purified TFIIIA interacts with a 5 S RNA gene relatively weakly (K_d 10^{-9}). TFIIC binds to the specific TFIIIA-5 S DNA complex via both protein-protein contacts with TFIIIA and protein-DNA contacts with the ICR (Pieler *et al.*, 1987; Wolffe, 1988; Hayes *et al.*, 1989). Together, TFIIIA and TFIIC bind so tightly to a somatic 5 S RNA gene that they do not dissociate even if free factor molecules are removed from solution or if excess competitor 5 S DNA is used to challenge the complex. The stability of the entire transcription complex, including TFIIB, reflects that of the TFIIIA-TFIIC-5 S DNA complex.

In contrast to the stabilization following the association of TFIIC with TFIIIA bound to a somatic 5 S RNA gene, little or no change in the binding of TFIIIA to an oocyte 5 S RNA gene follows the binding of TFIIC. The oocyte and somatic 5 S RNA genes differ in 5 bp within 120 bp of sequence, mainly in the ICR.

Surprisingly, TFIIIA alone binds with equal affinity to the somatic and oocyte type 5 S RNA genes (McConkey & Bogenhagen, 1988). Therefore the changes in sequence between the oocyte and somatic 5 S RNA genes have a much greater effect on the sequestration (or action) of TFIIC than on TFIIIA binding. The stability of the whole transcription complex on the oocyte 5 S RNA gene again reflects the stability of the TFIIIA-TFIIC-5 S DNA complex.

A major consequence of the differences in transcription complex stability on somatic and oocyte 5 S RNA genes is that conditions that limit transcription factor (TFIIIA and TFIIC) activity, concentration, or availability will selectively restrict transcription from the unstable oocyte 5 S RNA gene transcription complex. One obvious candidate for sequestering TFIIIA is 5 S RNA, hence the possible autoregulatory role of the TFIIIA-5 S RNA complex. This potential autoregulatory role for 5 S RNA has been tested. Oocyte and somatic 5 S RNA genes can both be programmed with transcription complexes in an extract of oocyte nuclei in which transcription factors are abundant. If an extract of eggs in which functional transcription factors are limiting is mixed with the pre-programmed genes, oocyte 5 S RNA gene transcription is selectively inhibited. Increasing the concentration of TFIIIA or TFIIC restores oocyte 5 S RNA gene transcription (Wolffe, 1989a). What removes these transcription factors from the oocyte 5 S RNA gene remains to be defined (Wolffe & Brown, 1987). Purified 5 S RNA added to the preprogrammed genes had no effect on differential transcription, seemingly excluding any direct autoregulatory role. Having postulated a mechanism for the regulation of 5 S RNA gene expression on the basis of *in vitro* experiments it is necessary to test this hypothesis *in vivo*.

Experiments *in vivo* with 5 S RNA genes and TFIIIA

Xenopus embryogenesis is characterized by eleven rapid cycles of cell division following fertilization (Fig. 3). This cleavage of the egg occurs in the absence of transcription. RNA synthesis in the oocyte nucleus ceases during the breakdown of structures such as the nuclear envelope on maturation into an egg. The developmental stage at which gene activity begins again is called the 'mid-blastula transition'; the embryo at this time has 4000 cells. At the mid-blastula transition, equivalent low levels of somatic and oocyte type 5 S RNA are synthesized. Because of the large excess of oocyte over somatic 5 S RNA genes, this represents a 50-fold higher rate of transcription for somatic than for the oocyte 5 S RNA genes. Two or three cell divisions later, near the end of gastrulation (10000–20000 cells), the final state of differential gene expression is established in which the oocyte 5 S RNA gene is almost completely repressed.

Various attempts have been made to examine the transcription factor and primary sequence requirements necessary to reproduce this developmental regulation by using cloned 5 S RNA injected into *Xenopus* eggs, and by perturbing transcription factor levels in the embryo. The injection of cloned repeats of oocyte and somatic 5 S RNA genes demonstrated that sequences within the ICR were responsible for at least a 50-fold discrimination between oocyte and somatic 5 S RNA genes in *Xenopus* eggs (Brown & Schlissel, 1985). Elevating the level of TFIIIA in the *Xenopus* embryo by injecting either the purified protein or synthetic messenger RNA enhanced oocyte 5 S RNA gene transcription relative to that of the somatic 5 S RNA genes (Andrews & Brown, 1987).

Therefore a central proposal of the *in vitro* model, i.e. that TFIIIA concentration is important for differential 5 S RNA gene expression, was confirmed. Moreover, as the ICR is the most important region of the gene mediating differential gene activity and TFIIIA binds to the ICR of oocyte and somatic genes equivalently, TFIIC would appear to also have a key regulatory role *in vivo*. The binding of TFIIC would be facilitated by an increase in TFIIIA concentration. Without TFIIIA being bound, TFIIC cannot recognize a 5 S RNA gene specifically (Setzer & Brown, 1985). The activation of oocyte 5 S RNA gene transcription by TFIIIA is only transient (Andrews & Brown, 1987), further suggesting that other factors also influence 5 S RNA gene expression. The eventual repression of the oocyte genes is attributed to chromatin assembly (see below and Wolffe, 1989b).

Our understanding of the developmental regulation of 5 S RNA gene transcription (Fig. 4) is perhaps the most complete for any eukaryotic gene. Although this results in no small part from the simplicity of the 5 S RNA gene system, many experimental strategies in common use today were pioneered using these genes. Much remains to be understood; however, this system uniquely demonstrates how a combination of *in vitro* and *in vivo* experiments with *Xenopus* can provide a biochemical definition of a regulated developmental process. Research with *Xenopus* class I and class II genes that will be outlined in the next sections encourages expectations that other developmental switches can be understood in comparable molecular detail.

CLASS I GENES: A SIMPLE SYSTEM FOR DEFINING ENHANCER ACTION

One of the major unresolved questions in eukaryotic molecular biology concerns the molecular mechanisms by which enhancers exert their stimulatory effects on transcription at promoters. The study of *Xenopus* class I gene expression continues to make major contributions to resolving this enigma.

The isolation and cloning of *Xenopus* rRNA genes was again facilitated by the abundance of the amplified extrachromosomal

copies of rDNA in oocytes (Botchan *et al.*, 1977; Boseley *et al.*, 1979). It soon became apparent from experiments in which cloned rDNA was injected into oocyte nuclei that the spacer DNA between rRNA genes had an important role in determining the efficiency of transcription by RNA polymerase I (Moss, 1983; Busby & Reeder, 1983). Repetitive elements (60–81 bp repeats) are present within the intergenic spacer. These sequences act as orientation- and position-independent enhancers of transcription (Labhart & Reeder, 1984; De Winter & Moss, 1987). Key regulatory elements found at the true promoter of the ribosomal RNA gene are also present within the spacer (Fig. 5). RNA polymerase I will also initiate transcription at these sites. Furthermore, the repetitive elements (60–81 bp repeats) share an 80% identity with sequences in the promoter of rRNA genes from –80 to –120 relative to the start of transcription (Boseley *et al.*, 1979; Sollner-Webb & Reeder, 1979). Competition experiments, in which transcription from a plasmid containing only the promoter was inhibited by the simultaneous injection of a plasmid containing only the repetitive elements, suggested that a common protein bound to these two sequences. This factor, called TF1S (or x UBF), has recently been purified (Dunaway, 1989; Pikaard *et al.*, 1989).

The requirements for the initiation of transcription of *Xenopus* rDNA genes by RNA polymerase I are relatively simple. TF1S constitutes the DNA-binding transcription factor, which recognizes both the enhancer and the promoter. A second protein (SL1 in humans; Learned *et al.*, 1983) forms a stable complex with TF1S on the rDNA promoter. TF1S may organize the DNA in an interesting way. DNAaseI cleavage patterns of the TF1S–rDNA complex suggest that the DNA is wrapped around the protein, perhaps bringing other regulatory elements into the correct juxtaposition for maximum effectiveness (Dunaway, 1989). Precisely what component of the transcription complex is recognized by RNA polymerase I remains to be determined.

Considerable progress has been made towards defining the mechanism by which class I gene enhancers operate in *Xenopus*. The microinjection experiments described above that defined the spacer elements as enhancers have been reproduced *in vitro* using transcription extracts derived from *Xenopus* oocyte nuclei (Pape *et al.*, 1989). Using this system, the rDNA enhancer has been shown to act during the establishment of the transcription complex. Once transcription complexes are assembled, the enhancer is no longer required. This hypothesis, that enhancers facilitate the initial formation of transcription complexes, is further substantiated by preliminary work on class II genes in both *Xenopus* oocytes and mammalian cells (Mattaj *et al.*, 1985; Weintraub, 1988). These results are also consistent with microinjection experiments, demonstrating that although the enhancer and promoter elements need to be close together for transcription to be stimulated, they do not need to be physically linked (Dunaway & Droge, 1989). This simple *Xenopus in vitro* system clearly offers much promise for eventually resolving the precise molecular mechanism responsible for enhancer action.

Xenopus class I genes have a considerable unexploited potential for developmental studies. As mentioned previously, these genes are amplified during specific stages of oogenesis (Brown & Dawid, 1968; Gall, 1968; Bird & Birnstein, 1971; Kalt & Gall, 1974). Recent research has indicated a role for eukaryotic transcription factors in the initiation of replication (De Pamphilis, 1988). The role, if any, of particular sequence elements and transcription factors in this unique system is yet to be defined. New methods of introducing DNA into immature oocytes such as electroporation or by the use of ballistic particles have now made the molecular basis of rDNA amplification experimentally accessible. Both during and after amplification the rRNA genes are transcribed at very high rates to produce the 4 μ g of ribosomal

Fig. 3. Major events in the developmental biology of *Xenopus laevis* from the perspective of a molecular biologist

A temporal sequence is shown from left to right.

RNA accumulated in the mature oocyte (Brown, 1966). Like the 5 S RNA genes, transcription is inhibited when the nuclear membrane breaks down during oocyte maturation. Ribosomal RNA genes are actively transcribed once again at the late blastula stage of development (Fig. 3). This event is developmentally regulated, rather than a consequence of a general activation of transcription, because class I genes are activated considerably later than class II or III genes (Shiokawa *et al.*, 1981). How these changes in rDNA transcription are regulated remains to be discovered.

CLASS II GENES: TRANSCRIPTION FACTORS AND DEVELOPMENTAL SYSTEMS

The most significant and complex regulatory circuits of all eukaryotic genes concern those encoding mRNA. Several excellent reviews on this problem have appeared, emphasizing tissue-specific gene expression (Maniatis *et al.*, 1987; Mitchell & Tjian, 1989; Johnson & McKnight, 1989). In this section I will not attempt to mention comprehensively every *Xenopus* homologue of a class II transcription factor but will concentrate on a few examples indicating the power of *Xenopus* as a developmental system. At the conclusion, I will illustrate how other *in vitro*

systems derived from *Xenopus* eggs for chromatin assembly and DNA replication may contribute to the utility of *Xenopus* in elucidating the molecular basis of developmental decisions at the level of protein-nucleic acid interactions.

Y-box transcription factors, mediators of germ-cell specific transcription?

The most thorough study of the regulatory elements of a class II gene in *Xenopus* is the systematic dissection of the Herpes Simplex Virus (HSV) thymidine kinase promoter by McKnight and colleagues (McKnight & Kingsbury, 1982; Graves *et al.*, 1986). A key regulatory element was defined through transcription of deletion and point mutations in microinjected *Xenopus* oocyte nuclei at the so called CCAAT box of the HSV thymidine kinase promoter. However, a more extended 12-bp DNA sequence was required for maximal transcriptional activity: 5' CTGATTGGCCAA 3'. This motif is found in two copies within a *bona fide* *Xenopus* gene for the 70 kDa heat shock protein (hsp 70) (Bienz, 1984; Tafuri & Wolffe, 1990). The promoter of the hsp 70 gene is known to be constitutively active in oocytes (Bienz, 1984, 1986). Importantly, deletion experiments have defined this regulatory element as being absolutely essential for oocyte-specific transcription (Bienz, 1986). The transcription

Fig. 4. Developmental regulation of differential 5 S RNA gene expression

(a) Regulation *in vivo* of the oocyte and somatic 5 S RNA genes. Both the ratio of somatic to oocyte 5 S RNA gene transcription on a transcription rate per gene basis (upper panel) and the total amount of 5 S RNA gene transcription on a per nucleus basis (lower panel) are shown. Although both oocyte and somatic 5 S RNA genes are maximally transcribed during the earlier stages of oogenesis, the total amount of 5 S RNA transcription falls towards the end of oogenesis. Following oocyte maturation into an egg, there is no transcription of 5 S DNA during fertilization and cleavage until the mid-blastula transition (MBT), at which stage the embryo has 4000 cells. At this time 5 S RNA gene transcription gradually increases, while the proportion of transcription from the oocyte 5 S RNA genes gradually decreases. Towards the end of gastrulation the final ratio of 1000 transcripts of a somatic 5 S RNA gene to every transcript from an oocyte 5 S RNA gene is established and maintained. Overall 5 S RNA gene transcription falls during later embryogenesis and in some tissues somatic 5 S RNA genes may become partially repressed. (b) Model for developmental control of 5 S RNA gene transcription. This diagram summarizes the occupancy of major oocyte (open arrow) and somatic (filled arrow) 5 S RNA genes with transcription complexes or chromatin during oogenesis and embryogenesis. A complex is represented on each gene. The absence of arrows implies that the complex is stable; the presence of arrows that the complex is unstable. The end result is that, by late gastrulation, stable transcription complexes are assembled on somatic 5 S RNA genes and the oocyte 5 S RNA genes adopt a repressed chromatin structure.

Fig. 5. Structure of the *Xenopus* rRNA gene and spacer (Dunaway, 1989)

A schematic representation of the *Xenopus* rRNA gene (tinted red) including the coding sequences for 28 S and 18 S rRNA (hatched boxes). The start of transcription is indicated (arrow). The red boxes represent the repetitive elements (60–81 bp) that constitute the enhancer element in the intergenic spacer. The black boxes represent promoter elements. A region of sequence similarity to the repetitive element exists within the promoter (see the text).

factors interacting with this key regulatory element might be expected to play an important role in class II gene expression in oocytes and perhaps other tissues.

The transcription factors interacting with this regulatory element were cloned in *Xenopus* (Tafuri & Wolffe, 1990) using a technique in which radioactive DNA sequences are used to probe a bacteriophage expression library (Vinson *et al.*, 1988). These proteins, FRG Y1 and FRG Y2, were found to be homologous to a human protein YB-1 that interacted with an identical sequence called the Y-box in mammalian MHC class II gene promoters (Didier *et al.*, 1988). Therefore, numerous Y-box transcription factors probably exist in eukaryotic cells.

The *Xenopus* proteins FRG Y1 and FRG Y2 share a region of identity with the human protein YB-1 and with an *E. coli* cold shock protein (Tafuri & Wolffe, 1990; Wistow, 1990; Goldstein *et al.*, 1990). This is within the DNA-binding domain of these proteins (S. R. Tafuri & A. P. Wolffe, unpublished work). There is little similarity in the primary amino acid sequence of the C-terminus between the Y-box proteins. However a secondary structural organization is conserved (Fig. 6). The C-terminus may be broken down into modules of basic amino acids (+) separated by acidic amino acids (–). The acidic regions are predicted to form α -helices, whereas the basic regions are predicted to have little organized structure. The basic regions resemble protamines and might therefore be expected to bind to DNA (Warrant & Kim, 1978). However, mutational analysis of these proteins indicate that the C-terminus does not interact directly with DNA (S. R. Tafuri & A. P. Wolffe, unpublished work). Instead, this protein domain appears to be involved in protein–protein contacts (Fig. 6). These protein–protein contacts might explain the requirement for a Y-box sequence element to be present at the hsp 70 promoter, in order that an enhancer containing a Y-box sequence can stimulate transcription at this promoter (Bienz & Pelham, 1986). Perhaps interaction between the Y-box proteins stabilizes the looping out of the intervening DNA.

In vitro transcription systems for class II genes in *Xenopus* have been developed (Tafuri & Wolffe, 1990; Corthesy *et al.*, 1990). Depletion of a *Xenopus* egg extract for the Y-box binding proteins selectively inhibits transcription from a *Xenopus* hsp 70 promoter; reconstitution with bacterially expressed proteins (either FRG Y1 or FRG Y2) reconstitutes active transcription from this promoter. These results demonstrate that these proteins

Fig. 6. Structure of the Y-box transcription factors

(a) The proteins may be divided into two functional domains, one at the N-terminus that is required for DNA binding, and one at the C-terminus that is required for protein–protein interactions (multimerization). The DNA-binding domain contains a highly conserved region homologous to *Escherichia coli* cold shock proteins (red). The C-terminus contains alternating regions with basic (+) or acidic (–) charge; the basic regions (tinted boxes) are protamine-like (see the text). (b) A potential means of protein–protein interaction for the Y box proteins.

are positive transcription factors, a conclusion consistent with the *in vivo* role of the Y-box *cis*-acting element.

FRG Y1 and FRG Y2 genes are differentially expressed during development. FRG Y2 mRNA accumulates only in immature oocytes and in the testis (Fig. 3; Tafuri & Wolffe, 1990). FRG Y1 mRNA accumulates in all tissues. It is especially interesting that a representative of the Y-box family of transcription factors binds a germ-cell-specific regulatory element and displays a germ-cell-specific pattern of expression. The many advantages of *Xenopus* for studying germ-cell-specific transcription encourages experiments to decipher the molecular mechanisms regulating FRG Y2 gene expression and the possible role of the FRG Y2 and FRG Y1 proteins in the developmental regulation of this gene.

Fig. 7. Regulation of transcription at the vitellogenin promoter (Corthesy *et al.*, 1989)

The vitellogenin B1 gene promoter contains two functional domains, a region required for oestrogen inducibility that contains binding sites for the oestrogen receptor (ORE, ▼). A more proximal region regulates transcriptional activity, this containing: (1) a TATA box, the key regulatory element for class II genes; (2) a binding site for a tissue-specific repressor (R); and (3) a binding site for a transcriptional activator (A). In the absence of oestrogen, the oestrogen receptor does not bind to the OREs; in this case the repressor binds to DNA and prevents the activator from functioning. When oestrogen is present, the receptor binds to the OREs, the repressor is prevented from functioning, and the activator stimulates transcription complex formation.

The oestrogen receptor: class II gene regulation *in vitro*

The first class II transcription factors to be identified and cloned in any organism were the steroid receptors (Evans, 1988). Research on the oestrogen regulation of vitellogenin (egg-yolk) gene expression in *Xenopus* liver have defined the key regulatory elements important for oestrogen-responsiveness and the mechanisms by which steroid receptors induce transcription (Seiler-Tuyns *et al.*, 1986; Klein-Hitpass *et al.*, 1986). Crude preparations of *Xenopus* oestrogen receptor isolated from liver were shown to be functional in activating endogenous vitellogenin gene promoters after microinjection into *Xenopus* oocyte nuclei (Knowland *et al.*, 1984). These studies were later extended to a functional dissection of the cloned human receptor (Theulaz *et al.*, 1988). The *Xenopus* oestrogen receptor has been cloned (Weiler *et al.*, 1987) and over-expressed such that abundant amounts of this transcription factor are available (Martinez & Wahli, 1989). Sequence analysis suggests that the oestrogen receptor contains zinc finger protein domains, as originally

defined in transcription factor TFIIIA. Most significantly for future progress in this field, *in vitro* systems are now available from *Xenopus* to dissect the action of the oestrogen receptor (Corthesy *et al.*, 1988).

The most transcriptionally active vitellogenin gene promoter *in vivo* has been most thoroughly dissected *in vitro* (Wolffe & Tata, 1983; Ng *et al.*, 1984). The vitellogenin gene B1 promoter has two regions containing key regulatory elements (Fig. 7). A distal region confers oestrogen inducibility, and a proximal region regulates transcriptional activity (Martinez & Wahli 1989; Corthesy *et al.*, 1989). The strong oestrogen-inducible enhancer is a modular unit composed of two imperfect 13 bp palindromic oestrogen-response elements. Alone, these elements confer only weak oestrogen responsiveness; together, they synergize to generate a strong response. The explanation for this phenomenon is that two oestrogen receptor dimers bind co-operatively to this pair of imperfect oestrogen response elements.

Hormone-controlled tissue-specific gene expression *in vitro* was first established for the *Xenopus* vitellogenin gene in *Xenopus* liver extracts. Dissection of the protein-DNA interactions in the proximal promoter element of the B1 gene using this system for functional assays has established that both negative and positive regulatory elements exist (Fig. 7; Corthesy *et al.*, 1989). The negative element binds a liver-specific transcription factor (repressor) and the positive element the *Xenopus* homologue of nuclear factor I (an activator; Jones *et al.*, 1987). In the absence of oestrogen, the vitellogenin gene is believed to be repressed through the binding of the liver-specific repressor. Hormonal induction requires the formation of the oestrogen receptor-DNA complex at the oestrogen response element that, in synergy with nuclear factor I, would relieve the inhibition due to the repressor. Depression would lead to the formation of an active transcription complex (Fig. 7).

One of the most interesting experiments involving the *Xenopus* oestrogen receptor concerns its autoregulatory role in controlling expression of the oestrogen receptor gene (Barton & Shapiro, 1988). The induction of the oestrogen receptor mRNA by oestrogen in male *Xenopus* hepatocytes results in the establishment of a stable, and possibly permanent, regulatory loop. The subsequent production of the oestrogen receptor protein maintains this autoregulatory loop long after oestrogen has disappeared from a male *Xenopus* frog. These results provide an experimentally accessible system for the study of irreversible developmental switches. Autoregulatory loops, first established with the oestrogen receptor representing a vertebrate transcription factor, may provide a simple mechanism to control the expression of key regulatory protein genes and thereby provide long-term modulation of the expression of numerous target genes (Barton & Shapiro, 1988).

Homeodomain proteins – a role in embryonic organization?

Many bacterial proteins regulating the transcription process contain a DNA-binding domain that has certain highly conserved features; this structure, known as the homeodomain, is also found in eukaryotic proteins (Gehring, 1987). These proteins, called homeodomain transcription factors, can exchange one segment of the body of *Drosophila melanogaster* into a different one (Lewis, 1978). The first vertebrate homeobox gene was cloned from *Xenopus* (Carrasco *et al.*, 1984). The *Xenopus* homeodomain proteins are believed to function in analogous ways to their *Drosophila* counterparts. However, definitive proof that they are transcription factors has not yet been established, although they offer useful markers and intriguing insights into developmental processes in the *Xenopus* embryo.

Several *Xenopus* homeodomain proteins have now been defined. The genes encoding these proteins are expressed ma-

ternally or early in development (Carassco *et al.*, 1984; Muller *et al.*, 1984), and some are induced by differentiation agents such as retinoic acid leading to a restricted spacial pattern of expression (Cho & De Robertis, 1990). *Xenopus* homeobox genes such as *Xhox-1* have been found to be activated at a precise stage in *Xenopus* development during early gastrulation (Fig. 3; Harvey *et al.*, 1986). the product of this gene appears to be involved in segmentation of the *Xenopus* embryo, for injection of synthetic *Xhox-1* mRNA disrupts somite formation (Harvey & Melton, 1988). The somites represent a segmental mass of mesoderm in the vertebrate embryo. Other *Xenopus* homeodomain proteins are involved in the growth and differentiation of the spinal cord (Sharpe *et al.*, 1987; Wright *et al.*, 1989).

More recent work on two other homeodomain proteins (*Xhox-3* and *Xhox-36*) has demonstrated distinct gradients of transcript accumulation in the *Xenopus* embryo (Condie & Harland, 1987; Oliver *et al.*, 1988; Ruiz i Altaba & Melton, 1989a). The appearance of particular homeobox gene transcripts in localized regions of the embryo is under the control of peptide growth factors (Ruiz i Altaba & Melton, 1989b). This strongly suggests that homeodomain proteins are the transducers that mediate cell commitment events signalled from the cell surface. Future work similar to that described for *Xenopus* class III genes and the vitellogenin genes will be required to dissect the molecular mechanisms by which homeodomain proteins regulate gene activation or repression, to exert what is clearly a central role in vertebrate embryogenesis.

***MyoD* – a regulator of muscle-specific gene expression?**

One of the first events in the organization of the *Xenopus* embryo is the induction of mesoderm, the cell lineage which will eventually give rise to muscle among other tissues (Gurdon, 1987; Smith, 1989). This process begins during the blastula stage and cell commitment is completed during gastrulation, when cardiac and skeletal muscle actin transcripts accumulate (Mohun *et al.*, 1984; Gurdon *et al.*, 1985). A great deal of attention has been focused on the molecular processes leading to actin gene expression, since these genes represent some of the earliest markers for tissue-specific gene expression during *Xenopus* development (Fig. 3).

Considerable progress towards understanding myogenesis has followed from experiments with mammalian cell lines (Davis *et al.*, 1987; Pinney *et al.*, 1988; Braun *et al.*, 1989; Wright *et al.*, 1989). Weintraub and colleagues have identified a gene, *MyoD*, whose expression can convert certain fibroblasts into myoblasts (Davis *et al.*, 1987). This finding has been exploited in *Xenopus* by workers attempting to determine the role of *MyoD* in muscle differentiation *in vivo*. *Xenopus MyoD* homologues have been cloned (Hopwood *et al.*, 1989; Scales *et al.*, 1990). These proteins are characterized by a DNA-binding and dimerization motif known as the helix-loop-helix domain (Murre *et al.*, 1989). Transcripts from the *Xenopus MyoD* genes accumulate very early in gastrulation, preceding the activation of cardiac-muscle-specific actin genes by over 2 h (Fig. 3). *MyoD* expression is restricted to the mesodermal lineage, and is eventually restricted to the developing somites from which muscle tissue will be created. Once the somites have formed, *MyoD* RNA concentration falls, consistent with a transient role for the *MyoD* protein in determining cell fate.

A direct test of the role of *MyoD* in determining cell fate has been made by microinjecting synthetic *MyoD* mRNA into early *Xenopus* embryos. The effect of this injection is to activate muscle genes inappropriately in embryonic cells normally destined to form a distinct cell lineage known as the ectoderm. In spite of this transient activation, these ectodermal cells fail to differentiate as muscle, suggesting that additional factors are required for

complete and stable myogenesis (Hopwood & Gurdon, 1990). This system therefore provides an excellent assay for these additional gene products.

Other developmentally regulated gene systems in *Xenopus* and DNA-binding proteins in search of system

Several other developmentally regulated gene systems are under investigation in *Xenopus*. In these systems, DNA-binding activities have been fractionated and in some instances the transcription factor cloned by homology.

The importance of the TFIIIA protein in regulating 5 S RNA gene expression was discussed earlier in this Review. The TFIIIA gene is much more active in oocytes than in somatic cells (Ginsberg *et al.*, 1984; Taylor *et al.*, 1986). This oocyte-selective expression may involve the Y-box transcription factors, as the TFIIIA gene 'CCAAT box' is important for transcriptional activation (Scotto *et al.*, 1988) and the FRG Y proteins will bind to this motif (S. R. Tafuri & A. P. Wolffe, unpublished work). However, a second transcription factor is also important for regulating TFIIIA gene expression. This protein (B1) is similar to the adenovirus USF or MLTF transcription factors (Hall & Taylor, 1989; Kaulen *et al.*, 1991). The gene encoding this protein has now been cloned (Kaulen *et al.*, 1991). Although the gene is expressed in both oocytes and somatic cells, distinct protein-nucleic acid complexes are formed using extracts from oocytes or somatic cells, suggesting that different forms of the protein exist. Future experiments will determine the nature of these differences and the consequences for regulation of the TFIIIA gene.

One of the most experimentally tractable cell lineages in *Xenopus* is the ectoderm. Ectodermal specific genes in *Xenopus* include the embryonal epidermal keratins. At the mid-blastula transition these genes are activated in a cell-autonomous process (Figure 3; Sargent *et al.*, 1986; Jamrich *et al.*, 1987). A transcription factor involved in this developmentally regulated process has been isolated (Snape *et al.*, 1990). This protein, KTF-1, binds to an essential regulatory element in the embryonic keratin promoter (Jonas *et al.*, 1989; Snape *et al.*, 1990). KTF-1 appears to represent the *Xenopus* homologue of the human transcription factor AP-2 (Williams *et al.*, 1988).

A period in *Xenopus* development with considerable unexploited potential for investigation using molecular techniques is amphibian metamorphosis (Tata, 1968). During this process (Fig. 3), the tissues present in the tadpole (larval stage) are completely restructured to form the froglet (adult stage). Metamorphosis has long been known to be dependent on thyroid hormone. Recently the thyroid hormone receptor genes in *Xenopus* have been cloned by homology to the human gene (Evans, 1988; Yaoita *et al.*, 1990). Representatives of two distinct gene families have been characterized: TR α and TR β . These genes are differentially regulated during development. Expression of both genes begins after the embryo hatches (Fig. 3). TR α mRNA accumulates before metamorphosis to a peak level actually at metamorphic climax and then falls. TR β mRNA only accumulates when the thyroid gland differentiates and presumably thyroid hormone is produced. The up-regulation of TR β mRNA is the earliest response to thyroid hormone so far detected in tadpoles (Yaoita & Brown, 1990). It is possible that the subsequent synthesis of the TR β thyroid receptor activates a cascade of regulatory factors that control the global morphological and biochemical changes characterizing the transition from tadpole to frog.

The advantages of *Xenopus* for both molecular biology and developmental studies of vertebrate embryogenesis encourage the idea that the cloning of any gene encoding a potential regulatory protein will lead into interesting developmental bi-

ology. The list of genes cloned on the basis of this philosophy continually grows. Most transcription factors have recognizable motifs, generally in the domain of the protein that binds DNA. There are zinc finger proteins (e.g. TFIIIA), helix–turn–helix proteins (e.g. homeodomain proteins), helix–loop–helix proteins (e.g. *MyoD*) and Y-box proteins (e.g. FRG Y1). Conserved DNA sequences encoding conserved amino acid sequences in these proteins can be used to clone homologues in *Xenopus*. Using this approach, 42 distinct cDNAs have been isolated containing zinc fingers (Nietfeld *et al.*, 1989). Additional *Xenopus* homologues of transcription factors include Oct-1 (Smith & Old, 1990), other members of this family (Baltzinger *et al.*, 1990), and ets-1/ets-2 (Stiegler *et al.*, 1990; Wolff *et al.*, 1990). The developmental significance of these proteins remains to be investigated.

A comparison of *Xenopus* to other organisms for investigating the developmental regulation of class II gene expression

Research on the regulation of class II gene expression in *Xenopus* is yielding fresh insights of general relevance to eukaryotic gene expression. Much of this progress follows from advances in methods of cloning transcription factors (Vinson *et al.*, 1988), the large stores of transcription factors in the immature oocyte and egg, and the ease of manipulation of the *Xenopus* oocyte and embryo. Research on *Xenopus* embryology does not employ genetic approaches and consequently large numbers of regulatory genes have not yet been isolated as they have in *Drosophila* (see De Pomerai, 1990). This is not necessarily a disadvantage as it is proving difficult to determine target genes for the abundant regulatory proteins in flies (other than the regulatory genes themselves). Regulatory proteins in *Xenopus* have in general been cloned by homology as they have in the mouse. Unlike the mouse, true transgenic experiments including gene disruption are not viable in *Xenopus* because of the long period to sexual maturity. However pseudo-transgenic experiments in which microinjected DNA is maintained in the embryo through to hatching are possible (Etkin *et al.*, 1984; Wilson *et al.*, 1986). This approach readily allows the definition of *cis*-acting elements that are important for correct temporal and spatial gene expression (Wilson *et al.*, 1986). It is also possible to alter the expression of regulatory molecules simply in *Xenopus*, either by injecting purified mRNA encoding the regulatory molecule or the regulatory molecule itself (Andrews & Brown, 1987). These issues are discussed in more detail below. However, an excellent example of convergent research in which the contribution of *Drosophila*, mouse and *Xenopus* is clearly illustrated concerns the role of the proto-oncogene *int-1* in development. In *Drosophila*, the *int-1* homologue encodes a gene (wingless, defined by standard genetic analysis) that is required in each segment of the fly to establish normal body pattern (Nusslein-Volhard & Weischaus, 1980). In the mouse, *int-1* expression is found by *in situ* hybridization to be restricted to a subset of neural cells in the embryo (Wilkinson *et al.*, 1987). In *Xenopus*, experiments can be performed to examine the significance of aberrant *int-1* expression (McMahon & Moon, 1989). Ectopic expression of *int-1* is shown to lead to duplication of the embryonic axis. Therefore important conclusions concerning the general role of *int-1* in determining the organization of an embryo follows from work integrating patterning events in vertebrate and invertebrate systems.

New methods are being developed that strengthen the utility of *Xenopus* in developmental studies of this type. As mentioned above, over-expression of a key regulatory molecule (or one with a role yet to be determined) can readily be shown to have both anticipated (and unexpected) results (Andrews & Brown, 1987; Harvey & Melton, 1988; Hopwood & Gordon, 1990; McMahon

& Moon, 1989). However, it has proved more difficult to ablate or remove stores of maternal RNA from oocytes or embryos by using antisense methods (Giebelhaus *et al.*, 1988). Recently, antisense oligonucleotides have been designed that are highly resistant to nucleolytic degradation, but that also serve as substrates for RNAase H, an endoribonuclease that specifically degrades the RNA strand of an RNA–DNA hybrid. RNAase H is abundant in *Xenopus* oocytes, adding to the utility of the system described below (Dash *et al.*, 1987; Shuttleworth & Colman, 1988). Non-ionic phosphate-modified oligodeoxyribonucleotides will form specific hybrids with mRNA in oocytes, leading to its degradation by RNAase H. The modified oligonucleotide can then rehybridize to a second mRNA and the stores of a particular mRNA are progressively eliminated (Dagle *et al.*, 1990). Synthesis of cyclin B1 and cyclin B2, components of *Xenopus* maturation-promotion factor (Gautier *et al.*, 1990) was inhibited by using this technique. As a direct consequence of eliminating cyclin mRNA, embryonic cell division ceased, demonstrating an essential role of the cyclins in the cell cycle *in vivo*. The success of over-expression and antisense ablation techniques can be expected to increase rapidly in the future.

The greatest utility of *Xenopus* as a system exists in biochemical analysis *in vitro*. The capacity to fractionate oocyte nuclei and egg cytoplasm (Birkenmeier *et al.*, 1978; Lohka & Masui, 1983) has facilitated the development of *in vitro* transcription systems for all three classes of eukaryotic gene (Birkenmeier *et al.*, 1978; Pape *et al.*, 1990; Tafuri and Wolffe, 1990; Corthesy *et al.*, 1990). Since both oocytes and eggs transcribe microinjected genes, correlations between experiments *in vivo* and *in vitro* are simple (McKnight & Kingsbury, 1982; Brown & Gurdon, 1978; Bending & Williams, 1984; Brown & Schlissel, 1985; Sollner-Webb & McKnight, 1982). These systems, first pioneered in *Xenopus*, have allowed the reconstruction of developmental regulation *in vitro* (Wolffe & Brown, 1988). The importance of multiple transcription factor–DNA interactions in assembling transcription complexes has also been realised from these studies (Brown, 1984).

It is increasingly recognized that transcription factors represent a necessary but not sufficient component of eukaryotic gene regulation. Work in yeast has clearly demonstrated that the chromosomal context of the gene will make important contributions to regulating transcriptional activity (Grunstein, 1990). Biochemical experiments in *Xenopus* have also suggested that developmental changes in chromosomal organization can influence 5 S RNA gene expression (Wolffe, 1990b). For example, histone H1 plays a key role in the repression of the oocyte type 5 S RNA genes (Schlissel & Brown, 1984; Wolffe, 1989b). Conversely, nucleosomes can potentiate vitellogenin gene activation (Corthesy *et al.*, 1990). Chromatin assembly systems are best documented for *Xenopus*; developed originally by Laskey and colleagues (Laskey *et al.*, 1977), they have been intensively used to examine the consequences for gene function of assembling a chromatin template (Almouzni *et al.*, 1990, 1991). Several aspects of eukaryotic gene regulation have so far failed to be reconstituted *in vitro* from fractionated components, e.g. enhancer function on class II genes. It is tempting to speculate that the missing component is some aspect of chromatin structure—perhaps chromatin facilitates the folding of the intervening DNA between enhancer and promoter. Preliminary experiments suggest that chromatin does indeed have a role in class II gene regulation (Workman *et al.*, 1991). It is reassuring that an entire nucleus can be assembled on exogenous DNA in a *Xenopus* egg or egg extract (Forbes *et al.*, 1983; Blow & Laskey, 1986). This demonstrates that more complex aspects of nuclear architecture with unknown functional implications, beyond arrays of nucleosomes, can be successfully reconstituted.

An important and unresolved question concerning eukaryotic gene expression concerns the molecular mechanisms responsible for maintaining stable states of gene expression (Brown, 1984). Transcription factors, chromatin structure and DNA replication are all proposed to play a role in the determination or commitment of a cell to a particular developmental fate. A unique aspect of *Xenopus* molecular developmental biology is the capacity to replicate somatic nuclei *in vitro* (Blow & Laskey, 1986). It is therefore possible to test the contribution of each of these components in determining gene activation and repression (see Wolffe & Brown, 1986; Almouzni *et al.*, 1991). No comparable systems currently exist for mouse or *Drosophila*.

CONCLUSIONS

The developmental biology of *Xenopus laevis* has many attractive features for a molecular embryologist. Although its genetics are less studied than with other systems, *Xenopus* has outstanding *in vitro* systems for biochemical analysis and a unique potential for surrogate genetics via the microinjection of macromolecules. Global changes in gene expression are well documented, but their precise molecular basis remains to be uncovered. Oogenesis represents a developmental period in which complex patterns of gene activity arise and disappear. Maturation of the oocyte into an egg inhibits all gene activity. This quiescence of the genome continues until the mid-blastula transition (Newport & Kirschner, 1982a,b) when all of the cell lineages begin to demonstrate their identity (Gurdon, 1987). Later, the entire larval form is restructured to the adult form during metamorphosis (Tata, 1968). All of these phenomena are open to investigation. Discoveries concerning the molecular mechanisms involved in *Xenopus* have proven to have general utility for all eukaryotic systems, and the future offers enormous scope for the fusion of the dissection *in vivo* of the significance of transcription factors in developmental pathways with the analysis *in vitro* of how these proteins function at the molecular level. The power of molecular biology and molecular genetics suggests that much of what we will learn about vertebrate development is likely to come first from research with *Xenopus*, as it has in the past.

NOTE ADDED IN PROOF

Since the preparation of this Review, several important articles on *Xenopus* transcription factors have appeared. These include: a description of regulatory proteins on the TFIID promoter [Pfaff, S. L. *et al.* (1991) Dev. Biol. **145**, 241–254], an analysis of CCAAT box binding proteins during embryogenesis [Orsenek, N. *et al.* (1991) Dev. Biol. **145**, 323–327] and the cloning of SRF [Mohun, T. J. *et al.* (1991) EMBO J. **10**, 933–940] and of AP-2 [Winning, R. S. *et al.* (1991) Nucleic Acids Res. **19**, 3709–3714].

I thank my colleagues in the Laboratory of Molecular Embryology, NICHD, for their comments on the manuscript. I am grateful to Ms. Thuy Vo for its preparation.

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