UNIVERSITAT DE BARCELONA

Modulation of transcription of sea urchin histone genes by a nuclear protein fraction: studies on the specificity of this activity using the amphibian oocyte microinjection assay

Susana Balcells Comas

MODULATION OF TRANSCRIPTION OF SEA URCHIN HISTONE GENES BY A NUCLEAR PROTEIN FRACTION: STUDIES ON THE SPECIFICITY OF THIS ACTIVITY USING THE AMPHIBIAN OOCYTE MICROINJECTION ASSAY

Memòria presentada per SUSANA BALCELLS i COMAS per aspirar al grau de Doctor en Biologia per la Universitat de Barcelona.

Treball realitzat en el Departament de Genètica del M.D.Anderson Hospital and Tumor Institute a Houston, Texas, sota la direcció del Dr. LAURENCE D. ETKIN.

Barcelona, Octubre 1987

V.P. V.P.

Susana Balcells i Comas

Dr.Laurence D. Etkin
Director

Dr.Jaume Baguñà i Monjo
Ponent

DEDICATORIA

Al Pau,

A la Teresa, la Pepa i la Hélène,

A la meva família, també

ACKNOWLEDGEMENTS

I would like to thank Dr. Lawrence Etkin, academic advisor, for providing me with the opportunity of pursuing research, for his encouragement and his support.

I would also like to thank Dr.David Wright, Dr. Donald Robberson, Dr. John Stein and Dr. Grady Saunders, for their guidance.

I would like to acknowledge: Dr. Eric Eastman, whose expertise was of great value to my project; Martha French who prepared the sea urchin protein fractions; and the Rosalie B.Hite Committee who awarded me a graduate fellowship in research to work on this project.

I want to thank also the people in the laboratory, in the Genetics Department,and elsewere in the Texas Medical Center: they constitute a powerful and rich cientific community and a friendly environment.

ABSTRACT

The early and late histone genes from the sea urchin Strongylocentrotus purpuratus constitute a pair of developmentally regulated genes. The early genes are expressed throughout oogenesis and early development until the blastula stage while the late genes are expressed at low levels until blastula and at higher levels thereafter. Cloned early and late histone H2b genes are transcribed upon injection into Xenopus laevis oocytes. Maxson et al. (1986), have shown that a protein fraction obtained from sea urchin gastrula chromatin stimulates the transcription of both early and late genes when it is injected into oocytes along with them.

I have used the technique of primer extension to characterize how several cloned genes are expressed in injected oocytes. The results show that chloramphenicol acetyltransferase (CAT) gene sequences are transcribed from multiple aberrant sites of the pSV2CAT plasmid as efficiently as from the correct start site. On the other hand, the sea urchin histone L1H2b gene and sea urchin Spec 1 gene are transcribed mainly from their correct start sites. Herpes simplex virus thymidine kinase (HSV tk) gene transcripton is also correctly initiated and the amount of transcripts produced is very high as compared to the other genes.

I have further studied the effect of the protein fraction from sea urchin gastrula chromatin on the expression of L1H2b gene injected into Xenopus oocytes, focusing on analyzing its specificity. The results show that this fraction contains a stimulatory activity for the transcription of L1H2b gene. The stimulation is reproducible and its magnitude is 10 fold on the average. I have also shown that the stimulation is specific for L1H2b and it does not affect either HSV tk or Spec 1 genes.

The activity described may be attributed to a transcription factor that is involved in the developmental regulation of the histone genes in the sea urchin.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

I. Transcription of Eukaryotic Genes

a) Genes work according to a set of signals and programs.

The theory of variable gene activity states that all cells in an organism retain an intact copy of the genome and that each cell type attains its phenotype by expressing a particular subset of genes. There must be coded information for the temporal and spatial regulation of genes. In other words, there has to be a program for gene expression in development as well as a program for gene expression inside each differentiated cell. It is the goal of developmental biologists to decipher these programs which at present remain largely unknown.

b) Genes can be isolated and cloned; they can be studied at the molecular level.

In the last 30 years, with the advent of molecular approaches, it has been possible to study the fundamental mechanisms involved in gene expression. We know that in all living cells genes are transcribed into RNA molecules and these are then translated into protein. Recent genetic engineering techniques permit the isolation and cloning of individual genes from complex genomes. The cloned genes can also be modified (by deletions, inversions, point mutations) and artificially expressed *in vivo* or *in vitro*.

Therefore, it is possible to study an isolated gene in a well defined environment and try to eventually reproduce step by step what happens in its wild type state.

c) Transcription is the major step where regulation of gene expression occurs.

Transcription and translation are multi-step processes in the pathway of gene expression and mechanisms of regulation may occur at any of these steps.

In 1961 Jacob and Monod used bacterial systems to study the molecular basis of gene expression since these were more amenable to study than complex eukaryotic systems. From their pioneering work it was discovered that regulation in bacterial operons (groups of genes under coordinate control) occurs mainly at the transcriptional level. Molecules that modulate transcription at an operon turn out to be proteins which in turn are encoded by other genes (which are called regulatory genes). Transcription of some operons is negatively controlled or repressed by regulatory proteins, whereas transcription of other operons is positively controlled or induced (Jacob and Monod, 1961).

In eukaryotic organisms gene expression is also regulated primarily at the transcriptional level. Modulation of transcription is known to acompany processes of development and differentiation. In the case of the fruit fly (Drosophila) it is known that in the salivary

glands of the larvae, the growth hormone ecdysone induces specific puffs in the polytene chromosomes (Ashburner et al. 1973). Puffs are sites of active transcription. So, part of the developmental program of Drosophila consists of releasing a growth hormone that triggers specific transcription. In the case of the hen, it is known that transcription of the ovalbumin gene is specifically induced by the hormone estradiol in the differentiated cells of the oviduct (Harris et al. 1975). In this case, specific activation of transcription accompanies a specific differentiated state of a cell.

d) The mechanism of transcription as we know it today.

Genes in eukaryotes are often composed of exons (coding sequences) and introns (non coding sequences). Genes in general have a promoter (site for recognition by the transcription machinery) most often upstream from the gene, a site of initiation of transcription and a termination signal at the end. RNA polymerases guide the base pairing between monomer ribonucleotides and DNA, catalyze phosphodiester bonding along the new RNA strand and are involved in initiation and termination of RNA synthesis.

There are three different eukaryotic RNA polymerase enzymes that transcribe three different groups of genes. Genes that encode proteins are transcribed by RNA polymerase II, ribosomal genes are transcribed by RNA polymerase I

and 5 S RNA genes and tRNA genes are transcribed by RNA polymerase III. The fundamental mechanisms of transcription are the same for the three enzymes but they differ in their recognition of specific promoter sequences.

e) Cis-acting sequences.

If a gene's transcription is to be regulated, we can imagine that there must be some genetic elements associated with it that respond to environmental signals controlling both the rate and fidelity of transcription. When such genetic elements lie on the same DNA molecule as the gene whose transcription they affect, they are referred to as cis- acting sequences. The sequencing and analysis of promoter regions of many genes has revealed that many promoters have an element called the TATA box, with a consensus sequence 5'TATAAA 3' located 25 to 30 base pairs (bp) upstream from the transcription start site (reviewed by Breathnach and Chambon, 1981). Mutations in this sequence generate 5' end heterogeneity in the transcripts although the overall level of RNA synthesis is not significantly reduced (Grosschedl and Birnstiel, 1980). Further upstream is a region containing one or more additional promoter elements. Some of these elements, such as the "CCAAT" (Benoist et al. 1980) or the "GGGCGG" homologies (reviewed by Dynan, 1986) have been found in many different promoters, whereas other elements such as the heat-shock regulatory element have a more specialized

role (Pelham, 1982). Upstream promoter elements are principal targets for the action of promoter specific transcription factors. Promoter activity may be modulated by an additional element called an enhancer. Enhancers lie on the same DNA molecule as the gene they affect but can act at distances of 1000 bp or more from the promoter and they may be located upstream or downstream from the transcription start site; also, their activity is independent of orientation on the DNA. The prototypical enhancer is found in the simian virus 40 (SV40) and consists of a 72 bp repeat sequence that lies upstream from the early promoter and enhances transcription from it (Banerji et al., 1981).

f) Transcription factors.

Environmental signals that affect the expression of a gene have been investigated by fractionating chromatin proteins from the tissue in which a particular gene is expressed and assaying the fractions for any modulatory effect on the transcription of that gene. Purified proteins obtained in this manner that modulate transcription are called transcription factors. In general, any molecule that affects the expression of a gene while working in trans is referred to as a trans-acting factor.

A well-characterized transcription factor that is required for the transcription of 5 S RNA genes is TFIIIA (transcription factor A of RNA polymerase III). This

factor may be used in the developmental control of the two kinds of 5 S RNA genes of Xenopus laevis. The evidence for this is that TFIIIA has different binding affinities for the oocyte type genes and for the somatic type genes (Wormington et al. 1981). Affinity for the oocyte type genes is low whereas affinity for the somatic type is high. During oogenesis TFIIIA is at a very high concentration in the oocyte (Shastry et al. 1984) and it interacts with the eight thousand copies of the oocyte type genes and these are transcribed. Later in development, TFIIIA gets diluted into the many cells generated by the cleaving embryo. In this situation it may no longer interact with the oocyte type genes. It is known that these genes adopt a stably repressed configuration and are not expressed. In somatic cells only the somatic type genes are being transcribed. Their stronger affinity for TFIIIA may account for their transcription in somatic cells where the factor is less abundant (reviewed by Brown, 1984).

The best known transcription factor for RNA polymerase II is Sp1. This factor, described and purified by Briggs et al. (1986), binds to the GGGCGG elements found in the SV40 early promoter region and stimulates early transcription of the viral genome. Sp1 also appears to play a role in the activation of other viral and cellular promoters such as Herpes simplex virus thymidine kinase gene (HSV tk) or mouse Dihydrofolate reductase gene (DHFR).

(reviewed by McKnight and Tjian, 1986).

Other transcription factors have been described that exhibit more gene specificity. These include transcription factor for a Drosophila heat shock gene (Parker and Topol, 1984), and another for human histone H4 gene (Heinz and Roeder, 1984).

These promoter specific factors represent potential candidates for sequence specific DNA binding proteins that differentially regulate gene expression. Indeed, partially purified factors have been tested for their ability to bind directly to specific promoters and in many cases they appear to do so. However, binding to DNA is not essential for specific transcription factors. For instance, some may act by modifying sequence specific repressor or activator proteins that are synthesized constitutively.

g) Transcription initiation complexes.

The step by step picture of how transcription is initiated on a pol II gene is not known yet. Work has been done fractionating a whole cell transcription extract into the essential components needed for accurate transcription from the Adenovirus major late promoter (AMLP) (reviewed by Dignam et al. 1983) and some preliminary kinetic studies have been done (Hawley and Roeder, 1985); it is known that transcription factors bind to DNA at the promoter to form preinitiation complexes that will transcribe upon addition

of pol II and ribonucleotides. At least four factors are required and these seem to be incorporated sequentially into the transcription complex.

At the present time theories on transcription regulation propose that cis-sequences and trans-factors join to form complexes of DNA and protein, and are held together not only through protein-DNA interactions but also through protein-protein interactions; for example, cis-sequences (such as enhancers) that lay at a distance from a promoter can act by binding a trans-factor that in turn recognizes and binds to a protein-DNA complex formed at a promoter. This binding will bring the two sequences close together in a complex, and make the intervening DNA form a loop (see Ptashne, 1986).

h) Systems that can be used to study transcription.

Three main kinds of systems have been developed and used for the study of eukaryotic gene expression. The first includes several cell-free extracts that allow accurate initiation of transcription of specific pol II genes in vitro. The second kind is the technique of transfection of cloned DNAs into cells by Ca^{++} precipitation. The third kind is microinjection of cloned genes into amphibian oocytes.

The cell-free crude extracts are made by lysis and homogenization of cells, separation of organelles and

membranous structures and collection of a crude mixture of soluble material. Cell-free extracts that can support transcription of pol II genes have been prepared (Weil et al., 1979; Manley et al., 1980). They can be used as the starting material for the fractionation and isolation of all the components required for the transcription of a gene. Upon fractionation of an extract, transcriptional activity may be lost and it can be regained by combining certain factors and not others. The cell-free extracts have the advantage that the investigator has more control over the factors required for the transcription reaction. The drawback of these systems is that they do not contain all the in vivo elements and so the information that we gain from them can be incomplete.

Eukaryotic DNA can be introduced into cultured mammalian cells by a procedure that involves Ca^{++} precipitation of the DNA and the subsequent phagocytosis of the calcium phosphate/DNA coprecipitate by the cells (reviewed by Gorman, 1982). A characteristic of this technique is that genetic markers are necessary to identify and selectively amplify the cells with the integrated DNA against a background of unmodified cells. Thymidine kinase- mutant cells have been used as hosts for Ca^{++} precipitated mixtures of a given gene cotransfected with the tk gene. Selection of tk^{+} cells is possible using HAT medium. Other dominant markers have been used that act on wild type cells

(as opposed to mutant cells); these include bacterial XGPRT gene that confers survival to cells cultured in mycophenolic acid and xanthine, and the bacterial neomycin resistance gene which confers resistance to cells grown in G418 (neomycin analog).

DNA can also be stably introduced into cells by direct microinjection into the cell nucleus using a glass micropipet. Microinjection requires special equipment to pull micropipets and a micromanipulator to hold them. It involves the use of a microscope and the manual injection of every single cell. The advantage of this technique is that any DNA can be injected and there is no need to apply a selective pressure to maintain the transferred gene into the cell. Microinjection into amphibian oocytes is relatively easy compared to microinjection into cultured cells, since oocytes are very large cells (1.4 mm diameter).

II. The Oocyte Microinjection System

Xenopus laevis is a frog species that has been used extensively by embryologists because both oogenesis and embryogenesis are accessible to study: oocytes are present in the ovaries of adult females at any given time in all the well characterized stages of oogenesis and in large amounts. Oocytes can be isolated individually or in large masses. Maturation can be induced in vitro. Synchronized embryos can be obtained in fairly large

amounts by fertilizing eggs in vitro. The developmental stages can be followed very well and they have been described (Niewkoop and Faber, 1967). Because fully grown oocytes are large single cells (1.4 mm diameter) they can be easily microinjected with a variety of substances. Zygotes and embryos can also be microinjected with DNA to study gene activity in early development (Etkin et al. 1984; Etkin and Balcells, 1985; Etkin and Pearman, 1987) and to obtain transformed animals (Etkin and Roberts, 1983).

a) Physiology of the oocyte.

During oogenesis, oocytes synthesize and store a variety of molecules to be utilitzed in early embryogenesis: they store yolk precursor protein and convert it to yolk (Wahli et al. 1981), they synthesize proteins, ribosomes (Brown and Littna, 1964), mitochondria (Dworkin et al. 1981) and they transcribe rRNA, tRNA, hnRNA mRNA and sn RNA (reviewed by Davidson, 1986). This metabolic activity and storage account for the structure and physiology of the fully grown oocyte (stage VI of Dumont, 1972). Full-grown oocytes are arrested at the diplotene stage of the prophase of the first meiotic division. The nucleus is very large (400 um diameter) and is called the germinal vesicle (GV). It contains about ten thousand times more core histones (Adamson and Woodland, 1974), RNA polymerases I, II and III (Roeder, 1974), and ribonucleotide triphos-

phates (Woodland and Pestell, 1972) than somatic cells. In the cytoplasm, ribosomes are ten thousand times more abundant than in somatic cells (Brown and Littna, 1964). In stage 6 oocytes no DNA synthesis occurs whereas genes are being actively transcribed. Oocytes injected with DNA at this stage undergo no visible change and can be maintained in culture for several days during which time they continue to synthesize RNA on the injected templates.

b) Microinjection into Xenopus oocytes.

Microinjection into a single cell was first performed using Rana pipiens eggs. The technique was used by Briggs and King in 1952 for nuclear transplant experiments which revealed important information concerning the pluripotency of somatic cell nuclei and contributed to the formulation of the variable gene activity theory mentioned above.

At the present time, applications of the technique include microinjection of DNA, RNA, proteins, small precursor molecules and organelles. Almost all the steps in gene expression have been shown to occur on injected molecules: naked DNA is assembled into chromatin and transcribed and primary transcripts are processed (reviewed by Gurdon and Melton, 1981; Etkin and DiBerardino, 1983); mature transcripts are translocated to the cytoplasm and translated; proteins follow their appropriate processes of modification, secretion and compartimentalization (reviewed

by Colman et al. 1981). Of particular interest for the work presented here is the finding that nuclear proteins injected into the oocyte's cytoplasm enter and accumulate in the GV (Bonner, 1975b).

Thus, it appears that the oocyte is amenable for microinjection of all kinds of molecules and that it is a metabolically active cell in which injected molecules are functional. The fidelity and efficiency of these processes varies widely for the different molecules injected and this may reflect that the oocyte itself indeed maintains a certain specificity in gene expression.

c) Transcription of injected genes into oocytes.

In the last 10 years microinjection of cloned genes into Xenopus oocytes has been used as an assay system in which to study transcription. Injected oocytes can transcribe a wide range of sequences and they can do so with more or less fidelity and efficiency. To a certain extent they represent an in vivo test tube for transcription.

Genes that have been tested for transcription into Xenopus oocytes include genes transcribed by each of the RNA polymerases (I, II and III). Genes from viruses, sea urchin, Drosophila, Xenopus, man and mouse that code for histones (Probst et al. 1979), tk (McKnight et al. 1981), alcohol dehydrogenase (Etkin et al. 1984), globin (Bendig and Williams, 1984), heat shock proteins (Voellmy and

Runnger, 1982) and myc (Nishikura et al. 1985), among others, are transcribed in the oocyte.

When DNA is delivered into the GV, its conformation is modified by endogenous components and becomes complexed with oocyte nuclear proteins on minichromosomal sructures, that is, it gets assembled into typicial nucleosome structures (Wyllie et al., 1978). Using electron microscopy it has been observed that only a fraction of injected molecules is being transcribed (forms active transcription complexes with RNA polymerases) while the majority of molecules appear inactive (Trendelenburg et al.,1978). Ryoji and Worcel (1984) have suggested that histones compete with other non-histone chromatin proteins for binding to DNA. The former will form `static' inactive minichromosomes whereas the latter will form `dynamic' actively transcribed minichromosomes. It has also been observed (Wyllie et al.,1978; Harland et al., 1983) that linear molecules delivered into the GV fail to be transcribed or do so at efficiencies several times lower that their circular counterparts. This may reflect the fact that linear molecules are unable to maintain torsionally stressed conformations whereas circular molecules can and do so; template topology thus appears to influence the transcription of DNAs injected into oocytes and may do so in general in any genome.

The results regarding fidelity and efficiency of

transcription of the different genes injected into oocytes show that some genes are transcribed mainly from their correct transcription start sites (e.g. the HSV tk gene, McKnight et al.,1981) while others generate a small proportion of correctly initiated transcripts (e.g. the sea urchin histone H1 and H4 genes, Hentschel et al., 1980). The efficiency of transcription varies from one gene to another but most genes introduced into the GV in super-colied conformation appear to be transcribed (that is, transcripts can be detected with the sensitivity of the assays used).

The oocyte microinjection system has been used as a transcription assay to assess the role of DNA sequences in the vicinity of a gene on its transcription. Grosschedl and Birnstiel (1980), constructed a series of 5' deletion mutants of a sea urchin H2a gene and assayed them for their ability to promote transcription of sea urchin H2a gene in oocytes. Results indicated that the TATA box is involved in specifying the site of initiation of transcription at a fixed position 25 to 30 bp downstream from it and that sequences upstream from the position -185 are involved in efficiency of transcription.

In similar experiments McKnight and Kingsbury (1982), mutagenized the upstream sequences of HSV tk gene using a linker scanning technique that replaces single bases with other bases without deletion. Mutants were also

assayed for transcription into oocytes. The results showed that the TATA box is involved in determining accurate initiation, whereas sequences 50 to 100 bp upstream from the transcription start site that contain two GGGCGG repeats and a CCAAT box play a role in the efficient transcription of the gene.

Finally, microinjection into oocytes has proven to be a viable system in which to assay the effect of trans-acting molecules on the transcription of a gene since both the gene and the molecule can be introduced into the oocytes by microinjection. Birchmeier et al. (1984), have described a trans acting factor that is necessary for the production of accurate 3' termini of the sea urchin H3 gene transcripts made in injected oocytes. The factor has been identified as a small poly A- RNA. Jones et al. (1983), co injected the E1A gene product from adenovirus and a construction carrying the adenovirus early promoter into Xenopus oocytes, and were able to reproduce the stimulatory effect of E1A on transcription from this promoter.

Mous et al. (1985) have described the stimulation of transcription of sea urchin early histone H2b genes injected into Xenopus oocytes by a chromatin protein fraction.

Maxson et al. (1986) have found that a fraction of sea urchin gastrula chromatin proteins contains a stimulatory activity for the transcription of sea urchin

early and late histone H2b genes injected into Xenopus oocytes (see below).

III. Sea Urchin Histone Genes

a) Developmental regulation of the expression of the sea urchin histone genes.

In the early development of the sea urchin Strongylocentrotus purpuratus two sets of histone genes are being expressed to produce the histones needed for packing the newly synthesized DNA. The two sets of genes differ in their developmental pattern of expression and also in their genomic arrangement location and copy number.

The early genes are expressed during oogenesis and early embryogenesis until blastula stage and they become silent thereafter. On the other hand, the late histone genes are transcribed at very low levels during early cleavage stages, their transcription rate increases by blastula stage, and their higher rate of transcription is maintained thereafter (Childs et al. 1979). During early cleavage and blastula, the histones that pack the newly synthesized DNA are contributed mainly by the expression of early genes whereas after blastula, there is a switch and the histones are mainly contributed by the expression of the late genes.

Early and late histone genes are arranged differently in the genome. The early genes are present at about 400 copies per genome (Weinberg et al. 1975) and are

clustered in tandemly repeated units each containing one copy of each histone type gene (Kedes et al. 1975). By contrast, the late genes are present at 5 to 12 copies per genome and these are not clustered together (Maxson et al., 1983).

In previous work Maxson et al. (1986), the differential expression of early and late histone genes was analyzed. It was hypothesized that at gastrula there might be a factor that would stimulate transcription of the late H2b gene only. The hypothesis was tested by fractionating sea urchin gastrula chromatin and testing three fractions (0 - 0.45 M NaCl wash, 0.45 - 1.0 M NaCl wash and 1.0 - 2.0 M NaCl wash) for their modulatory activity on injected sea urchin early and late H2b gene transcription.

The protein fractions were injected into the cytoplasm of oocytes several hours ahead of the DNA in order to allow their migration into the nucleus. DNA (either early or late H2b genes) was then injected into the GV and allowed to transcribe. Early or late H2b specific messages were then assayed by using appropriate S1 probes and compared to those produced in the absence of gastrula chromatin proteins.

One of the fractions tested (0.45 - 1.0 M NaCl wash) appeared to contain a stimulatory activity for both the early and late H2b gene transcription but the magnitude of the stimulation was 5 times larger for late genes as

compared with early genes. Late H2b gene transcripts were 10 times more abundant in the presence of the fraction than in its absence. By contrast the level of early gene transcripts was stimulated only 2 fold by the presence of the fraction.

This thesis describes the work that I have done to characterize the activity of this protein fraction, and to test whether this activity is specific for the H2b genes of sea urchin. The results section is divided in three main parts. In the first part I tested my accuracy of micro-injection into the oocyte's GV, to measure the sensitivity of the microinjection assay. In the second part I describe some of the different ways of studying gene expression. As an example, CAT gene expression is studied both at the transcriptional and translational levels. Also, a detailed study of the primer extension analysis of the transcription of several genes is presented. The genes tested were sea urchin L1H2b, sea urchin Spec 1 gene, SV2CAT gene and the Herpes simplex virus thymidine kinase gene. The primer extension analyses of these genes were a prerequisite for the third part of the results. In this last part, the characterization and study of the specificity of the activity of the protein fraction are presented. The characterization includes a dose response analysis of the amount of protein fraction injected and a study of the time course of preincubation of the protein in the oocyte. The

study of specificity includes the tests of the effects of the fraction on TK gene and Spec 1 gene transcription.

MATERIALS AND METHODS

I. Recombinant Plasmids

a) Description of the recombinant plasmids pSV2CAT, pSpL1, pSpec1.1 and ptk.

The characteristics and diagrams of the recombinant plasmids used are in table I and figure 1 respectively.

pSV2CAT (Gorman et al., 1982) is a plasmid that contains the simian virus 40 (SV40) early transcription region into which a chloramphenicol acetyltransferase (CAT) gene of E.coli has been inserted. The SV40 early transcription region is thus split into a promoter region located 5' to the gene and a polyadenylation signal region located 3' to the gene. The promoter region is 340 base pairs (bp) long and contains the early promoter, the 72 bp repeat enhancer and the origin of replication. The vector for pSV2CAT is a 2.3 kilobase (kb) long fragment of pBR322 which contains the Ampicillin resistance (Amp^r) gene and the origin of replication of the plasmid.

pSpL1 (Maxson et al., 1983) contains one copy of the late histone H2b gene and one copy of late histone H4 gene in a 9.5 kb stretch of sea urchin S.purpuratus DNA. The late H2b gene is 1.5 kb downstream from the late H4 gene, and has about 6 kb of 3' flanking sequences. The vector for pSpL1 is the plasmid pBR322. The insertion is

between the EcoRI and HindIII sites.

pSpec1.1 (Hardin et al. 1985)) is a plasmid that contains a portion of the Spec1 gene (4.3 kb) from the S.purpuratus subcloned in the SalI site of vector pUC8 (2.7 kb). The 4.3 kb insert is a XhoI restriction fragment that contains the promoter, the first exon (0.2 kb)and a portion of the first intron (1.38 kb) of the Spec1 gene, as well as 2.7 kb of upstream sequences. The whole construction is 7 kb long.

ptk/ 3'- 1.13/ 5' -182 (McKnight et al. 1981) is a plasmid that carries one copy of the thymidine kinase gene (tk) from herpes simplex virus (HSV). The gene is in a 1.8 kb fragment and is flanked by 182 bp of HSV DNA upstream and 294 bp downstream. The vector is the plasmid pBR322, and the cloning site is at the BamHI and HindIII sites. The whole construction is 5.8 kb. This plasmid will be referred to as pTk.

b) Transformation procedure.

Transformation of E.coli with the different recombinant plasmids was done according to Hanahan (1983), with some modifications, as follows. One colony of E.coli HB101 was picked from a frozen stock and dispersed in 4 ml of L Broth (10 g Difco tryptone, 5 g Difco yeast extract, 5 g NaCl in 1 L water; pH to 7.4 by addition of $\tilde{}$ 5 ml 1 M NaOH; 1 ml 1 M $MgSO_4$ and then sterilized by autoclaving). After overnight (O/N) growth at 37^{o} C, the culture was

diluted 100 X (0.46 ml of culture into 46 ml fresh L Broth in a 250 ml flask) and incubated at 37° C on a shaker (275 revs/min) until A_{550} = 0.45 ($\tilde{}$ 2 h). The cells were collected into a 50 ml polypropylene tube and pelleted at 2500 rpm for 12' at 4°C. The cells were resuspended gently in 20 ml of wash buffer (Peacock et al., 1981) (20 mM sodium acetate, 0.1 M NaCl, 5 mM $MgCl_2$, pH 5.6) and pelleted again; the pellet was resoftened very carefully, resuspended in 20 ml of transformation buffer (Norgard et al., 1978) (40 mM sodium acetate, 70 mM $MnCl_2$, 30 mM $CaCl_2$, pH 5.6), placed on ice-water for 20' and pelleted by centrifugation at 1000 X g for 12' at 4°C. The pellet was carefully resuspended in 2 ml of transformation buffer. Cells (100 µl) were placed into chilled 17 mm x 100 mm polypropylene tubes and 2 µl (1 ng) of each transforming DNA was added to each sample. The mixtures were swirled and incubated on ice-water for 30'. Tubes were heat-shocked at 42°C for 90 seconds and placed on ice-water for 1' to 2'. Then, 0.9 ml of L Broth (at room temperature (RT)) was added and tubes were incubated at 37°C on a shaker at 200 rev/min for 1 h. Appropriate fractions of each culture (20µl, 50µl and 100µl) were spread onto Ampicillin plates using a glass rod. Plates were incubated at 37°C to establish colonies.

c) Plasmid preparation and purification.

Single colonies from the transformation reaction

were analyzed to assess that the bacteria were indeed transformed (that they carried the appropriate plasmid)by performing "minipreps" (alkaline lysis procedure of Birnboim and Doly (1979) as modified by Ish-Horowicz and Burke (1981).. After this, a glycerol stock of the cells was made and stored at -80°C. Large preparative extractions were made from the stock following the same alkaline lysis procedure.

Minipreps. Several colonies were picked from the transformation plate and grown in polypropylene tubes in 5 ml of L Broth containing ampicillin. Cultures were allowed to grow O/N at 37°C with vigorous shaking. 1 ml of cells was separated in an eppendorf tube, 0.15 ml of sterile glycerol added and stored at -80°C as a stock. The remaining 4 ml were analyzed for the presence of the plasmid by the alkaline lysis procedure described below but scaled down 100 X and with the following additional steps. The last pellet of nucleic acid was resuspended in 50µl of TE Buffer pH 8.0 containing 20 µg/ ml DNase-free RNase, and a 10 µl aliquot was digested with the proper diagnostic restriction enzyme. Digested as well as non digested DNAs were electrophoresed on agarose gels along with the corresponding control plasmids. Gels were stained with EtBr and photographed under UV light.

Alkaline lysis method for preparative extractions of plasmids from transformed bacteria. Bacteria from the

stock were streaked on an ampicillin plate and a single colony was picked to inoculate 5 ml of L Broth + ampicillin in a polypropylene tube. The tube was incubated O/N at 37°C with vigorous shaking and diluted 100 X (2.5 ml of culture in 250 ml L Broth + ampicillin in a 1 L flask). The growth of this large culture was monitored every 30' by measuring the OD at 660 nm, until it reached 0.5. At this point plasmids were induced to amplify by addition of chloramphenicol to a final concentration of 170 µg/ ml and incubation was resumed O/N. Cells were pelleted at 3500 X g at 4°C for 10' and resuspended in 10 ml of solution I (5 mg/ ml Lysozyme, 25 mM Tris-HCl pH 8.0, 10 mM EDTA and 50 mM glucose) in a polyallomer tube. After 5' at RT, 20 ml of fresh solution II (0.2 N NaOH, 1 % SDS) was added, tubes were mixed gently and were incubated on ice for 10'. Finally, 15 ml of ice-cold solution III (5 M potassium acetate pH 4.8 that is 3 M potassium acetate + 2 M acetic acid) were added and sealed tubes were inverted sharply several times and incubated on ice for 10' to allow the proteins, SDS and chromosomal DNA to differentially precipitate. Tubes were centrifuged at 16000 rpm on a Beckman JA 20 rotor for 30' at 4°C. The supernatant containing the plasmid DNA was transferred to Corex tubes. Plasmid was precipitated by addition of 0.6 vol of isopropanol (or 2 vol of ethanol) for 15' at RT, and pelleted at 12000 X g for 30' at RT. Pellets were rinsed

with 70 % ethanol, dried and resuspended in 8 ml TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA).

Cesium chloride (CsCl)-ethidium bromide (EtBr) gradients. preparations were centrifuged through a CsCl-EtBr density gradient so as to separate closed circular supercoiled plasmid DNA from RNA, chromosomal DNA and proteins (Maniatis et al. 1982). 1 mg solid CsCl/ 1 ml of preparation was added and dissolved. 0.1 ml EtBr (10 mg/ ml)/ 1 ml of original preparation was also added and well mixed. Ultracentrifugation was at 45,000 rpm at 20°C for at least 36 h. The closed circular plasmid band was identified by UV illumination and collected by puncturing the tube with a syringe.

EtBr was removed by 4 to 6 extractions of water saturated 1-butanol until no color remained in the aqueous solution. The sample was then diluted 4 X with deionized H_2O, precipitated in ethanol and reprecipitated to remove excess CsCl. The final pellet was brought up in a small volume of TE; OD measurements were made to calculate yield, concentration and quality of the DNA preparation. 1.0 OD_{260} is equivalent to 50 µg/ ml of DNA. An OD_{260}/ OD_{280} ratio of 1.8 is indicative of DNA separated from proteins. Agarose gel electrophoresis of the final plasmid preparations was done to check the identity and integrity of the plasmid.

Purification of plasmids by Sepharose column

chromatography (Just et al. 1983). To completely separate plasmid DNA from small RNA molecules, plasmid preparations were loaded onto a column of Sepharose 6B (Pharmacia) (2.5 x 20 cm) in 1 X STE buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl and 1 mM EDTA). Column loading buffer was 10 % glycerol and .04 % bromophenol blue. Fractions were collected and their absorbancy was monitored at 260 nm. The plasmid DNA was collected as a first peak separated completely from a second peak of RNA. The fractions comprising the peak were pooled and precipitated in 0.3 m sodium acetate by addition of 2.5 vol of cold ethanol. After centrifugation at 12000 X g the pellet was rinsed in 70 % ethanol and resuspended in TE buffer. OD measurements were taken and the concentration was adjusted to 1 µg/ µl.

II. The Ocyte Microinjection System

a) Procurement and handling of oocytes.

Surgery on Xenopus females: adult Xenopus laevis females were anesthesized by hypothermia in crushed ice for 30 minutes (30'). An abdominal incision was made in one side of the animal to obtain lobes of ovarian tissue. A desired number of lobes was cut with scissors. The fragment of tissue was transferred to a dish that contained Modified Barth's Solution (MBS) (see below). The incision was closed by making one stitch with plain gut absorbable surgical suture (Ethicon #6-0). After the operation frogs were kept in water containing crushed ice to allow them to

return to room temperature (RT) gradually. They were kept under observation for one day to make sure that they were recuperating.

Defolliculation and care of oocytes. Oocytes were kept in MBS (88 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 10 mM Hepes pH 7.4, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$ and 0.41 mM CaCl) at all times.

The oocytes are surrounded by two layers of follicle cells. The follicle needs to be removed for ease of microinjection as well as to obtain single cell oocytes. Stage 5 and 6 oocytes (Dumont, 1972) were placed under a dissecting microscope and defolliculated manually with the aid of watchmaker's forceps. Defolliculated oocytes were kept in fresh MBS at 18° to 20°C and were used within the next 24 hours. Defolliculated oocytes are viable under these conditions for approxiamtely one week.

b)Preparation and calibration of glass micropipets

Micropipets were heat pulled from glass tubing (0.8 mm internal diameter, 1.0 mm outer diameter) using a Livingston micropipet puller. The occluded tips were opened using watchmaker's forceps and sharpened to a beveled tip using a deFonbrune microforge. The sides of the pipets were scored using the microforge to permit calibration.

A solution of ^{32}P was used to calibrate the pipets by filling them to the different marks. Duplicate volumes

from each mark were spotted on Whatmann glass fiber filters, and counted in a Scintillation counter by the Cerenkov method. A standard curve of 0.2, 0.5, 1 and 2 µl of a 100 X dilution of the ^{32}P solution was made. Cpms of 1 µl of the standard curve correspond to cpms of 10 nl of the spotted filters.

Micropipets were washed after each use by drawing a warm solution of 95% nitric acid 5% sulfuric acid through the tip; the acid was rinsed away by washing the pipets in deionized water about 10 times.

c) The microinjection technique.

Microinjection of oocytes was done under a dissecting microscope using a micromanipulator. Oocytes were placed in the wells of a plexiglass plate and covered with MBS. Samples for injection were placed as drops on the side wall of a well surrounded and covered completely by paraffin oil to avoid evaporation. Injections into the cytoplasm of oocytes were performed by introducing the pipet at the equatorial plane of the oocyte (at the edge between the pigmented animal pole and the unpigmented vegetal pole). Injections aiming at the GV were performed by introducing the pipet at the center of the animal pole along the animal-vegetal axis (see Figure 2). In general, a group of 12 oocytes was injected with each different sample.

The amounts of DNA injected into the GV varied

between 0.3 and 7 ng (see Results) and the volumes did not exceed 10 nl. The amounts of protein injected varied between 1 and 20 ng and the volumes did not exceed 40 nl.

Incubation of injected oocytes was in fresh MBS in petri dishes at a temperature of 18 to 20°C. Oocytes injected with protein were incubated for 5 hours prior to the injection of DNA; oocytes injected with DNA were incubated for 5 hours piror to RNA extracion or storage.

RNA extraction of fresh samples was preferred over storage of frozen oocytes since it resulted in RNA of slightly better quality. When immediate extraction of RNA was not possible, oocytes were collected into eppendorf tubes, drained from excess MBS and frozen in a dry ice-methanol bath. Oocytes were kept at -80°C until the extractions could be done.

III. CAT Assay

The CAT Assay was done according to (Gorman et al. 1982). Briefly, oocytes (individually or in groups of 5) were collected in 1.5 ml eppendorf tubes, 100 µl of homogenization buffer (0.25 M Tris-HCl pH 8.0) was added to each tube, and the samples were homogenized by pipetting buffer and oocytes in and out of the tip of the pipetter. Homogenates were then spun for 15' at 4°C in a microfuge and the clear supernatants (or a fraction of them) were collected for the assay. Assay reactions contained 0.02 uCi ^{14}C-Chloramphenicol, 3 µl of fresh 3 mg/ ml solution of

acetyl-CoA and control enzyme or homogenate samples in 100 µl of 0.25 M Tris-HCl pH 8.0. Incubations were at 37°C for 1 h. Extraction of the chloramphenicol and acetylchloramphenicol was done by bringing the volume of the samples up to 1 ml and adding an equal volume of ethyl acetate. The top organic phase was saved dried and resuspended in 20 µl of ethyl acetate. To separate the substrate chloramphenicol from the acetylated products, samples were spotted onto silica gel plates and chromatographed with chloroform-methanol (95:5) for 30'. Plates were exposed to X ray film at -80°C O/N.

The spots corresponding to the unreacted substrate and the product were cut, disolved in scintillation fluid and counted in a liquid scintillation counter. Enzyme activity was measured as percent conversion of the initial amount of substrate to the acetylated products by calculating: counts of the product ÷ (counts of the remaining substrate + counts of the product) x 100.

IV. Extraction of Nucleic Acids

a) RNA.

RNA was extracted by performing phenol and chloroform extractions of a homogenate of oocytes in an aqueous RNA extraction solution (0.3 M NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA and 2 % SDS) (Gurdon et al., 1983). Typically up to 12 oocytes were homogenized in 0.5 ml of RNA extraction solution. Two phenol (re-destilled and

neutralized) extractions, three phenol-chloroform (1:1) extractions and two chloroform-isoamylalcohol (24:1) extractions (and back-extractions) were performed until all material trapped in the interphase had disappeared. At this point nucleic acids were precipitated by addition of 2 volumes (2 vol) of cold ethanol and incubation at -20°C O/N. Precipitates were pelleted by centrifugation in an eppendorf centrifuge (~15000 x g) for 20', and pellets were rinsed with 70% ethanol to remove salts.

DNase I digestion. Pellets were resuspended in 100µl of 10 mM Tris, 0.1 mM EDTA (Low TE Buffer) and a small volume was saved for future analysis of the injected DNA. The rest of the nucleic acid was digested with DNase I enzyme (RQ1 DNase, RNase-free, from Promega Biotec.) to eliminate the plasmid DNA. Reactions were in 40 mM Tris-HCl pH 7.5, 60 mM $MgCl_2$, 10 mM DTT and 0.05 u of DNase I per ng of DNA, in a 200µl vol. (about 200 µg of nucleic acid / ml of reaction volume). Reactions were incubated for 30' at 37° and at this time one phenol-chloroform and two chloroform-isoamylalcohol extractions were performed to remove the enzyme. RNA was then precipitated by addition of 0.5 vol of 7.5 M NH_4 acetate (final concentration 2.5 M) and 2 vol of cold ethanol and kept O/N at -20°C or 20' at-80°C.

Estimates of the yields and concentrations of RNA by optical density measurements. Precipitates were

pelleted, washed with 70 % ethanol and resuspended in small volumes of Low TE. An aliquot of each sample was appropriately diluted and its Optical Density at 260 and 280 nm was measured. A ratio 260/280 of 2.0 was indicative of uncontaminated RNA. Concentrations were calculated using the conversion 1.0 unit of A_{260} equals a concentration of RNA of 40 mg/ ml.

Assesment of the quality of the RNA by running glyoxal denaturing gels (McMaster and Carmichael, 1977; Thomas, 1983). RNA samples (0.5 µg) were glyoxylated in 1 M glyoxal (deionized), 10 mM sodium phosphate buffer pH 7.0 and 50 % DMSO in a vol of 8 µl at 50°C for 1 h. Loading buffer (2 µl) (50 % glycerol, 0.5 % Bromophenol blue, 10 mM Sodium Phosphate Buffer pH 7.0) was added to each sample and these were loaded onto a 1.1 % agarose gel in 10 mM sodium phosphate buffer pH 7.0. Electrophoresis was carried out at at 50 Volt (50 V) with recirculation of the buffer until the bromophenol blue had migrated 2/3 of the length of the gel. The gel was then deglyoxylated in 50 mM NaOH for 30', neutralized in 20 mM sodium phosphate buffer 2 times 15' and stained in an EtBr solution (0.5 µg/ ml in 20 mM sodium phosphate buffer) for 1 h prior to visualizing the stained RNA on an ultraviolet (UV) transilluminator. A non degraded RNA preparation shows that 28S RNA band is twice as intense as the 18 S RNA band and both bands are sharp.

Aliquots and storage of the RNA. 5 µg aliquots of each sample were made before addition of 2 vol of cold ethanol and storage at -80°C.

b) DNA.

DNA samples from injected oocytes were obtained by the same procedure as RNA samples but no DNase I digestions were performed.

V. Southern Blots

a) Agarose gel electrophoresis of DNA.

Samples of DNA from injected oocytes (one oocyte equivalent) were run on 0.7 % agarose gels in 1 X TAE Buffer (40 mM Tris-acetate, 2 mM EDTA) at 50 V until the bromophenol blue tracking dye had migrated 3/4 of the length of the gel. Gels were stained in 0.5 µg/ ml EtBr for 15' and DNA was visualized and photographed on a UV transilluminator.

b) Transfer of DNA to membranes (Southern, 1979).

DNA was depurinated in 0.25 M HCl for 5' at RT with shaking. Next, DNA was denatured in 0.5 M NaOH containing 1.5 M NaCl twice for 15' and neutralized in 1 M Tris-HCl pH 8.0 containing 1.5 M NaCl once for 20'. Gels were placed on filter wicks connected to a reservoir of 20 X SSC (3 M Na Cl and 0.3 M Na citrate). A charged nylon membrane (Genescreen brand) pre-wet and pre-equilibrated in 20 X SSC was placed on top of the gel, and the assembly was topped with filter paper and paper towels and covered with

plastic wrap with a weight on top. Transfer was carried out O/N. After transfer was complete, the gel was re-stained with EtBr to check that no DNA was remaining. The membrane was then removed from the blotting sandwich and baked between filter paper at 80°C in a vacuum oven for 2 h.

c) Nick-translation labeling of DNA.

Plasmid DNAs to be used as probes were radioactively labeleled by the nick-translation reaction (Kelly et al., 1970; Rigby et al. 1977). Reactions typically contained 0.5 µg of DNA in 50 µl of 20 mM each of dATP, dTTP, dGTP, 1 µM dCTP, 100 uCi ^{32}P-dCTP (3000 Ci/ mmol; Amersham), 50 mM Tris-HCl pH 7.5, 7.5 mM $MgCl_2$, 1 mM DTT, 50 µg/ ml nuclease-free BSA, 2.5 u of Kornberg DNA Polymerase I and 25 pg DNase I (optimal proportions of polymerase to DNase to generate labeled fragments of ≃ 500 nucleotides were previously tested by running the products of various reactions on alkaline denaturing gels). Reactions were incubated at 14°C for 1 h and stopped by addition of 2 µl of 500 mM EDTA. Unincorporated nucleotides were separated away from labeled DNA strands by centrifuging the reaction through a 1 ml Sephadex G-50 column. Specific activity was measured by Cerenkov counting of 1 µl of the reaction spotted on a DEAE filter. 4 x 5' washes with 0.5 M dibasic phosphate buffer effectively displaced the non incorporated nucleotides from

the filters and these were counted again. Specific activity was calculated as total retained counts (incorporated in the DNA)/ µg of DNA. Usually values of 10^8 cpm/ µg were obtained.

d) Hybridization and exposure of membranes.

Baked membranes were first prehybridized in a sealed bag in the following prehybridization solution: 5 X SSPE, 10 X Denhardt's, 1 % SDS and 0.5 mg/ ml salmon sperm DNA in a volume of 50 - 100µl/ cm^2 of membrane. 20 X SSPE is 3.6 M NaCl, 200 mM NaH_2 PO_4 and 20 mM EDTA; 50 X Denhardt's is 1 % Ficoll, 1 % Polyvinyl pyrrolidone and 1 % BSA. Prehybridization was carried out for 12 h at 65°C.

Nick-translated ^{32}P labeled DNA probe was used at 25 - 50 ng per ml of prehybridization mix. The probe was mixed with 0.5 ml of 10 mg/ ml salmon sperm DNA and denatured by boiling at 100°C for 5'. This mix was then added to the bag containing the membrane and the prehybridization mix. The bag was resealed and incubated at 65°C for 12 h.

Membranes were washed after hybridization to remove all the unbound labeled DNA probe. The following washes were performed: two rinses of low stringency solution (50 ml) inside the bag and at RT. Two 15' washes in low stringency solution (250 ml) in a box at 65°C. Several 15' washes in high stringency solution (250 ml) in a box at 65°C (as many as required to reduce the counts on

the membrane to background level). Low stringency solution is 2 X SSC and 0.5 % SDS; high stringency solution is 0.1 X SSC and 0.5 % SDS.

Washed membranes were exposed wet to fast film (Kodak XAR) with an intensifying screen O/N at -80°C. More High Stringency washes were performed if necessary, to reduce background signal.

VI. Dot Blots

RNA samples were denatured in 7.4 % formaldehide as described (White and Bancroft, 1982). To the RNA samples (in 50 µl of TE) was added 30 µl of 20 X SSC and 20 µl of 37 % formaldehyde, and the samples were then incubated at 60°C for 15'. The denatured samples (100µl) were applied with suction to 4 mm diameter spots on a nitrocellulose sheet (prewet in dH_2O and equilibrated in 20 X SSC) using a 96-hole Minifold apparatus (Schleicher and Schuell). The nitrocellulose sheet was then baked in a vacuum oven at 80°C oven for 2 h. Prehybridization of the nitrocellulose, preparation of nick-translated probes, hybridization and exposure was done as described in the Southern blot section.

VII. Primer Extension Analysis of RNA Molecules

The technique of primer extension is designed to analyze the fidelity of transcription. It requires the use of an oligonucleotide primer complementary to an internal portion of a specific mRNA near its 5' end. This piece of

single stranded DNA is then radioactively labelled and used in excess to hybridize to the desired mRNA in a total RNA mixture; then, it serves as a primer for the synthesis of a DNA strand complementary to the 5' end of the mRNA molecule, in a reaction carried out by the enzyme reverse transcriptase. The primer gets extended to a longer strand of DNA according to the length of the template mRNA. If three different start sites have been used by the oocyte transcription machinery, then the primer extension will result in three different species of extended primer that will be resolved as three different bands in acrylamide-urea denaturing gels; the intensity of each band will reflect the abundance of transcripts of each particular species.

a) Purification of synthetic oligonucleotide primers.

The following primers were synthesized chemically: a 20-mer for SV2CAT, a 24-mer for HSV TK, a 17-mer for early and late sea urchin histone H2b and a 15-mer for Spec (their characteristics are summarized in Table II).

Primers synthesized on solid support by the phosphoramidite (or phosphite triester) method (Caruthers, 1982; Atkinson and Smith, 1984) and delivered as a dry powder were resuspended in 80 % Acetic Acid and treated for 10' at RT to remove any traces of the trityl groups with which the 5' OH ends are protected during the synthesis

process (Warren Zimmer, personal communication). Subsequently, they were dryed and rinsed in sterile deionized water to remove traces of acid.

A preparative size denaturing gel of 20 % Acrylamide and 7 M Urea was used to separate the primers from smaller size oligonucleotide contaminants (Maxam and Gilbert, 1980). The gel was pre-electrophoresed at constant power (20 watt) (to warm it to 40-45^{o}C) in 1 X TBE Buffer (89 mM Tris-Borate, 89 mM Boric Acid, 2.5 mM EDTA).Primers were resuspended in loading buffer (98 % formamide, 1 mM EDTA, 0.01 % bromophenol blue and 0.01 % Xylene Cyanol) and warmed to 50 - 60^{o}C for 5' prior to loading. The gel was run at 20 - 30 watt constant power (550 - 650 V) until the bromophenol blue dye migrated 2/3 of the length of the gel.

Bands were visualized by UV shadowing. The gel was placed on a TLC UV fluorescent plate and illuminated with a short wave length UV lamp. Dark shadowed bands on the gel corresponding to the primers were cut using a clean razor blade, and pulverized inside eppendorf tubes using baked glass rods. To elute the primers from the acrylamide, 2 vol of 0.3 M Na acetate pH 5.0 were added and the tubes were sealed and incubated for 14 h at 37^{o}C with constant agitation. Tubes were spun and supernatants were recovered. Eluted primers were then extracted twice with a mixture of phenol - chloroform (1:1) and once with chloro-

form - isoamyl alcohol (24:1) and precipitated in 10 vol of cold ethanol (-80°C for 1 h). Following centrifugation the pelleted primers were rinsed with absolute ethanol prior to drying and resuspending in TE buffer. The yields of the primers were estimated by measuring the optical density (OD) of an aliquot at 260 nm and using the conversion 1.0 OD unit is equivalent to 35 µg/ ml of a single stranded oligonucleotide.

To check the quality of the primers, aliquots were ^{32}P end-labelled (see ahead) and electrophoresed on a sequencing type gel (0.4 mm thick and 30 cm long), 20 % acrylamide, 7 M urea, 1 X TBE Buffer. A second round of purification was necessary since the primers were still contaminated with a small amount of smaller size oligonucleotides. Re-purified primers were finally stored at -20°C.

b) <u>End-labeling of primers</u>.

5' end-labelling of primers was done according to (Maxam and Gilbert, 1980). The T4 polynucleotide kinase forward reaction for protruding 5' ends was used to label the primers for sea urchin H2b gene, HSV TK gene and Spec1 gene. 1 - 50 pmoles of dephosphorylated 5' ends were mixed with a 3 X molar excess of ^{32}P ATP (3000 Ci/ mmol) in 50 µl of kinase buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA and 10 - 20 units of

BMB T4 polynucleotide kinase). The reaction was carried out at 37°C for 30' and stopped by addition of 2 µl of 500 mM EDTA. Unincorporated nucleotides were separated from labelled primer through a Sephadex G-25 spun column: reactions were chloroform extracted once and brought up to 100 µl in STE Buffer ; then they were spun through a 1 ml Sephadex G - 25 column.

Calculations of specific activity, incorporation of label and recovery from the column were done by spotting 1µl of reaction mixture as well as 1µl of the material recovered from the column on DEAE filter papers. These were counted by the Cerenkov method, and subsequently washed 4 X 5' in 0.1 M Na_2HPO_4, 1 X 5' in dH_2O, and 2' in ethanol; filters were counted and dried again. The dibasic phosphate washes effectively remove unincorporated nucleotides, so that the counts in the washed filters measure radioactivity incorporated into the primers only, whereas the counts in the original filters measure the total radioactivity used. Specific activity is calculated as the ratio of total incorporated counts to the mass of primer used.

The primer for pSV2CAT gene was labelled using the forward reaction for blunt 5' ends. The CAT primer sequence is such that two base pairs can form between the last two nucleotides at each end. The 5' end to be labelled is thus in a blunt end configuration and requires

a preliminary heating step to open. 1-50 pmoles of primer were heated to 70°C in 40 µl of a buffer that was 20 mM Tris-HCl pH 9.5, 1 mM spermidine and 0.1 mM EDTA and chilled on ice. 5 µl of 10 X blunt end kinase buffer (0.5 M Tris-HCl pH 9.5, 0.1 mM $MgCl_2$, 50 mM DTT and 50 % glycerol) was added, as well as 3 X molar excess of ^{32}P-ATP ans 20 units of polynucleotide kinase. The reaction was incubated at 37°C for 30' and stopped by addition of 2 ml of 500 mM EDTA.

c) Hybridization of primers to RNA and reverse transcriptase reaction.

The procedure used was that of McKnight et al. (1981), with some modifications. In general, 1 ng of labelled primer was used as an excess amount to hybridize to a sample of 5 µg of total RNA from injected oocytes. Primer and RNA were mixed and dried together in a Savant speed - vac. Mixtures were resuspended in 10 µl of hybridization buffer (10 mM Tris-HCl pH 7.5, 0.3 M NaCl, 1 mM EDTA) and incubated for 2 h at a temperature between 5 and 10°C below the temperature of dissociation (Td) of the primer (Td = 4(G + C) + 2(A + T)) (Meinkoth and Whal, 1984) (Td values for each primer are listed in table II). The precise temperature was determined for every different primer by assaying both Td - 5 and Td - 10.

Extensions were performed by adding 10 µl of 10 X reverse transcriptase buffer (500 mM Tris-HCl pH 8.3 at

42°C, 1.2 M KCl, 80 mM $MgCl_2$ and 100 mM DTT); actinomycin D to a final concentration of 50 µg/ ml, each of the four deoxynucleotide triphosphates to a final concentration of 1 mM, and 10 units of BMB avian myeloblastosis reverse transcriptase to the hybridization reactions. The reaction volume was adjusted to 100 µl and incubation proceeded for 1 h at 42°C. Nucleic acids were then precipitated by addition of 50 µl of 7.5 M NH_4 acetate and 300 µl of cold ethanol O/N at -20°C.

d) Acrylamide-urea denaturing gel electrophoresis (Maniatis, 1982; McKnight et al. 1981).

Samples from the primer extension reactions were pelleted, resuspended in 20 µl of Loading Buffer (90 % formamide, 1 X TBE, 0.02 % bromophenol blue and 0.02 % XC) and denatured for 4' at 95°C prior to loading in the wells of a 10 % acrylamide (20 acrylamide : 1 methylen bisacrylamide) 7 M urea denaturing gel. Running buffer was 2 X TBE. Gels were electrophoresed at 5 watt constant power (≃ 220 V) until the bromophenol blue was at the bottom of the gel (bromophenol blue co-migrates with DNAs of 12 nucleotides in this percentage gel). Gels were fixed in 10 % methanol 10 % acetic acid for 30' and dryed in a gel dryer under vacuum for 1 h. Gels were exposed to fast film Kodak XAR with intensifying screen O/N at - 80 °C and subsequently exposed to Fuji film (blue, less background grain) for longer periods of time.

VIII. Preparation of Chromatin Protein Fractions

a) Preparation of nuclei from sea urchin gastrula.

Fertilized S.purpuratus eggs were grown as a 1 % (v/v) suspension in artificial sea water to the gastrula stage (25 -30 h at 14°C). Embryos were pelleted at 700 X g for 2', degellied in Ca^{++} and Mg^{++} free sea water (0.45 M NaCl, 50 mM KCl, 10 mM EGTA, 5 mM EDTA, 10 mM Tris-HCl pH 7.5) and pelleted again. Embryos were separated from smaller contaminating particles by resuspending the pellet in 1.5 M dextrose and centrifuging in a clinical centrifuge at full speed for 10' and then the cells were lysed in a low salt buffer (4 mM magnesium acetate, 50 mM Tris pH 7.5, 5 mM b-mercaptoethanol, 0.1 mM PMSF and 0.5 % NP40). Lysis was monitored by phase microscope observation. Nuclei were separated from cell debris by centrifuging the lysates through a 1.7 M sucrose cushion (containing 0.1 mM PMSF) at 8000 X g for 12'. Pelleted nuclei were stored frozen at -80°C.

b) Preparation and fractionation of chromatin.

Nuclei were lysed in 75 mM NaCl, 5 mM Tris and 25 mM EDTA, 0.1 mM PMSF, 0.5 mM DTT, homogenized and pelleted at 12000 X g for 12'. The chromatin pellet thus obtained was extracted first with an extraction buffer (20 mM Tris-HCl pH 7.0, 1 mM EDTA, 1mM EGTA, 0.5 mM DTT and 0.1 mM PMSF)that was .45 M in NaCl. Solubilized material was saved and constituted the first protein fraction (0 - 0.45

M NaCl wash). The remaining pellet was washed again with extraction buffer that was 1.0 M in NaCl, and the solubilized material was saved as the second protein fraction (0.45 M - 1.0 M NaCl wash). Finally the remaining pellet was washed with extraction buffer 2 M in NaCl, and the solubilized material was saved as the third fraction (1.0 M - 2.0 M NaCl wash). Fractions were dialyzed against 20 mM Tris, 10 % glycerol 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT 0.1 mM PMSF and 100 mM KCl, and spun to remove precipitated material (mostly histones). Protein yields were determined by the Bradford assay and the fractions were aliquoted and stored at -80^{o}C.

RESULTS

I. Determination of the Accuracy of Microinjection

DNA microinjected into the GV of Xenopus oocytes is known to persist and to be expressed, whereas DNA delivered into the cytoplasm is known to be degraded (Wyllie et al. 1978). GV injections can be done by penetrating the oocyte at the animal pole along the the animal-vegetal axis. It is important to assess one's accuracy of microinjection into the GV. This can be done by injecting a gene and either looking for its product or checking the integrity of the DNA.

a) CAT assays.

The CAT assay is an easy and quick test for gene expression. The CAT gene encodes the enzyme chloramphenicol acetyl transferase; this enzyme catalyzes the conversion of chloramphenicol into acetyl chloramphenicol. The assay consists of incubating the samples that may contain the enzyme with ^{14}C-chloramphenicol and acetylCoA. If the enzyme is present, a product ^{14}C-acetyl chloramphenicol is made, and this can be separated from the substrate by TLC and quantitated by counting the radioactivity of the spots (see materials and methods). The CAT gene has been linked to the SV40 early promoter in a recombinant plasmid pSV2CAT (Gorman et al. 1982) and this construction is expressed in Xenopus oocytes (Etkin and Balcells, 1985).

I tested the effect of the site of injection on the expression of CAT gene by injecting pSV2CAT plasmid into oocytes either at the equatorial region (cytoplasm) or at the animal pole (GV) (Figure 2). Single oocytes were assayed for activity of CAT enzyme (Figure 3). When pSV2CAT was injected at the equatorial region, no CAT activity could be detected (Figure 3 C). When the DNA was injected at the animal pole, 12 oocytes out of 13 oocytes that were assayed showed CAT activity, indicating successful delivery into the GV. In this experiment the accuracy of microinjection into the GV was 92 %.

b) Southern blot.

The integrity of the injected DNA was determined by performing Southern blots. Oocytes were injected with 1.6 ng of pSV2CAT plasmid either at the equatorial region or at the animal pole (see figure 2). DNA from single oocytes was then extracted, separated by electrophoresis on an agarose gel, transferred to a nylon membrane and hybridized to a radioactive probe of pSV2CAT plasmid (Figure 4). Injections of pSV2CAT in the equatorial region of the oocyte resulted in degradation of the DNA which appeared as a smear (Figure 4 C). Twelve oocytes were injected with pSV2CAT at the animal pole (Figure 4 D). The analysis of the recovered pSV2CAT DNA showed that in 10 oocytes it was intact, and in 2 oocytes it was degraded (Figure 4 D, lanes 5 and 7). The accuracy of injection

into the GV was 83 %.

The combined results of the CAT assays and the Southern blot indicate that 22 out of 25 (88 %) oocytes were accurately injected into the GV.

II. Tests of Expression of Injected Genes

Genes successfully delivered into the GV of Xenopus oocytes are known to be expressed. Expression of injected genes can be studied at various levels from transcription to translation. For those genes whose products are known enzymes, expression at the translational level can be done by assaying the samples for the enzyme activity: such is the case of CAT. When the protein product of a gene is unidentified or when we want to study transcription, expression is studied by analyzing the newly synthesized RNA. A simple method of analysis consists of spotting the RNA samples as dots on a membrane and hybridizing the membrane with a labeled probe of the gene whose expression we are studying (Dot Blot Analysis of RNA). By this method we detect the presence of a specific transcript and we get a rough estimate of its amount, but we do not get any qualitative information (size, initiation site or termination site).

The technique of primer extension described in Materials and Methods is a precise and sensitive method for the analysis of the 5' end of a specific RNA species. It gives us information on the fidelity of initiation of an

RNA molecule and it also can give us information on the abundance of this specific RNA in a sample. Primer extension is thus a tool of choice for the analysis of the mRNAs produced by foreign genes introduced into the GV of oocytes.

a) Expression of CAT gene.

I characterized three parameters of the expression of CAT the gene in the GV of Xenopus oocytes: 1) the dose response of the amount of injected pSV2CAT, 2) the time course of appearance of enzyme activity, and 3) the time course of appearance of CAT transcripts.

Dose response of the amount of pSV2CAT injected.

Groups of five oocytes were injected with increasing amounts of pSV2CAT ranging between 0.3 and 1.5 ng and were incubated for 24 h. CAT assays were performed and enzyme activity was measured (Figure 5-I and Table II). As a positive control of the assay, CAT activity was measured on known amounts of purified CAT enzyme (Figure 5-I, A). The results are shown in Figure 5-I,B: 0.3 ng of injected plasmid resulted in 1 % conversion (a barely detecatble signal on the autoradiograph) (lane 3); 0.6 ng resulted in 13 % conversion (lane 2), 0.9 ng resulted in 33 % conversion (lane 4 in Figure 5-I,C) and 1.5 ng resulted in 82 % conversion (lane 1). Therefore, a correlation between the amount of plasmid injected and the enzyme activity was observed. Detection of enzyme activity was

possible when $\geq$ 0.6 ng of plasmid were injected (0.6 ng of pSV2CAT represent 1.1x 10^8 copies of this plasmid).

Time course of enzyme activity. Groups of five oocytes were injected with 0.9 ng of pSV2CAT and incubated from 2.5 h to 24 h. Subsequently they were assayed for CAT enzyme activity (Figure 5 C). CAT enzyme activity was not detected for the first 10 h (lanes 1,2 and 3), but it was obvious at 24 h (lane 4). This experiment was repeated (not shown) and the same result was obtained. These data show that at least 10 h are necessary for the transcription and translation of the injected CAT gene.

Time course of appearence of CAT transcripts. Groups of oocytes were incubated for varying amounts of time following pSV2CAT injection, and the presence of CAT mRNA was assayed by Dot Blots. Total RNA was extracted and RNA samples from the different groups were denatured and blotted as dots onto a nitrocellulose membrane. The presence of CAT transcripts was detected by hybridization with a radioactive probe of pSV2CAT and autoradiography. Figure 5-II shows the results of two such experiments. In A, 5h (lane 2) and 10 h (lane 3) were tested and in both cases pSV2CAT specific mRNA was observed. In B a shorter time point of 2.5 h (lane 3) was analyzed and pSV2CAT specific mRNA could also be detected. Thus, CAT mRNA is present in the oocyte as soon as 2.5 h after injection of the gene. The CAT enzyme however, is not detectable until

at least 10 h.

b) Expression of genes injected in Xenopus oocytes; analysis at the mRNA level by primer extension.

The technique of primer extension was used to analyze transcription of the mRNAs specific for sea urchin L1H2b gene, CAT gene, sea urchin Spec 1 gene, and HSV TK gene. In order to study the best assay conditions for each mRNA species, it was desirable to obtain RNA samples in which the specific message was abundant. Therefore, a relatively large amount of each plasmid (4 to 7 ng) was injected per oocyte, and was allowed to transcribe for 24 h before RNA extraction. Primers specific for each mRNA were made and purified (see materials and methods). Important parameters of the primer extension assay such as absolute and relative amounts of primer and sample RNA, temperature and time of hybridization and amount of reverse transcriptase enzyme were tested in order to optimize the detection of the extension products.

Expression of sea urchin late histone H2b gene.

Sea urchin total RNA contains several species of H2b mRNA that vary in presence and abundance in the different developmental stages. These species of H2b mRNA are detected and resolved as a family of extension bands in a primer extension analysis using the sea urchin H2b specific primer (Figure 6, lanes 4 and 5). In egg RNA (lane 4) a

major upper band of 205 nucleotides was detected that represents early H2b mRNA. Two minor bands were also detected and these represent late H2b mRNA species. In sea urchin gastrula RNA (lane 5) two major bands and one minor band of 150 nucleotides just below them were detected; they represent three different species of late H2b mRNA. The band at 150 nucleotides represents the mRNA transcribed from late 1 H2b gene (L1H2b) which is the gene used in my injection experiments. There is also an upper band of 205 nucleotides that corresponds to early H2b mRNA.

To test for the expression of injected LH2b gene, 4 ng of pSpL1 were injected into the GV of oocytes and these were incubated for 24 h. RNA was extracted and analyzed by primer extension using the end labeled primer specific for sea urchin H2b mRNA (Figure 6, lanes 6,7,8 and 9). As negative controls primer extension reactions were performed using 5 µg of tRNA (lane 2) or 5 µg of non injected oocyte RNA (lane 3).

Two extension bands were detected in lanes 6-9 that were not detected in the control lanes, and they were identified as L1H2b specific. The lower band (150 nucleotides), co-migrates with the minor species of wild type sea urchin LH2b mRNA, and thus represents a population of LH2b transcripts that were correctly initiated when transcribed by the oocyte's transcription machinery. The upper band (~ 205 nucleotides) co-migrates with the wild type early H2b

mRNA, but actually represents a sub population of aberrant LH2b transcripts that were initiated some 50 bp upstream from the correct initiation site. This band can be resolved from the early HD2b mRNA band by changing the urea concentration of the gel and running the gel for a longer period of time.

To find the best proportions for the primer extension assay the absolute and relative amounts of primer and sample RNA were changed and tested . In experiment 1 increasing amounts of primer (40 pg, 400 pg and 1 ng) were hybridized to 5 µg of total oocyte RNA from pSpL1 injected oocytes (Figure 6, lanes 6 - 8). It was observed that the intensities of the bands specific for LH2b transcripts increased proportionally to the amount of primer and were strongest at 1 ng of primer. In experiment 2 different amounts of sample RNA (5 µg in lane 8 and 1 µg in lane 9) were tested with 1 ng of primer. When only 1 µg of RNA was hybridized to 1 ng of primer, the signal for LH2b mRNA dropped to a faint band (Figure 6, experiment 2: compare lane 9 to lane 8).

From the results of experiments 1 and 2 it was determined that the best signals were obtained by hybridizing 5 µg of total RNA from injected oocytes with 1 ng of the specific primer.

Expression of CAT gene. To test for the expression of CAT gene 7 ng of pSV2CAT plasmid were injected into the

GV of oocytes and these were incubated for 24 h prior to RNA extraction; total RNA was hybridized to an end labeled CAT specific primer and primer extension reactions were performed (Figure 7).

The CAT specific primer used hybridizes 123 nucleotides downstream from the 5' end of SV2CAT mRNA. An extension band of 123 nucleotides is expected to appear if transcripts from the injected CAT gene are initiated properly. In all lanes containing injected oocyte RNA (Figure 7, lanes 3-11), the patterns of extension products were rather complex and comprised a family of bands rather than one major extension band. None of these bands was present in control lanes containing tRNA (lane 1) or non injected oocyte's RNA (lane 2). This indicated that all the extension bands were CAT specific mRNA products. The multiple bands reflected a complex pattern of initiation of transcription on the injected pSV2CAT plasmid.

Different temperatures and times of hybridization were tested to find the best conditions for the primer extension reactions. Three different temperatures were tested: 45^{o}C (Figure 7, lanes 3,6 and 9) is Td - 15; 50^{o}C (lanes 4,7 and 10) is Td - 10; and 55^{o}C (lanes 5,8 and 11) is Td - 5. Three different times of hybridization were tested at the same time: 1 h (lanes 3,4 and 5), 2 h (lanes 6,7 and 8) and 4 h (lanes 9, 10 and 11). Within each time point, the intensities of the extension bands increased as

the temperature of hybridization increased and the strongest signals corresponded to the experiments in which the temperature was 55°C (Td - 5). Within the 55°C group and across the different times of hybridization (lanes 5, 8 and 11) the intensities of the bands increased from 1 h (lane 5) to 2 h (lane 8) and did not change between 2 h (lane 8) and 4 h (lane 11) of incubation. The general trend observed indicates that 55°C (Td - 5) and 2 or more hours of hybridization result in optimal primer extension signals.

Expression of sea urchin Spec1 gene. Expression of injected sea urchin Spec1 gene was studied by primer extension (Figure 8). Oocytes were injected with 7 ng of pSpec1.1 plasmid and incubated for 24 h prior to RNA extraction and primer extension analysis with an end labeled Spec 1 specific primer.

A sample of 10 ng of sea urchin gastrula RNA was included in the primer extension analysis (lane 3). The extension products of the Spec primer on total sea urchin gastrula RNA appear clustered and have lengths around 55 nucleotides (a band at 55 nucleotides was identified as the correctly initiated Spec1 mRNA (Susan's ref.)).

Extension products of the Spec1 specific primer on injected oocyte RNA are shown in figure 8, lanes 4 - 7: the major product was a doublet which migrated as two of the wild type Spec 1 specific extension products in lane 3. No

extension product of this size could be detected in control primer extension reactions with tRNA (lane 1) or non injected oocyte RNA (lane 2). The doublet was thus identified as the correctly initiated Spec 1 transcript. A longer and fainter extension band that migrates at 110 nucleotides was also present; such a band was not detected in the analysis of the control RNAs either; it may represent a transcript aberrantly initiated some 50 bp upstream from the true start site in the plasmid.

Several parameters of the primer extension technique were assayed: temperatures of 38°C (Td - 10) (Figure 8, lane 4) and 43°C (Td - 5) (lane 5) were compared; time of reverse transcriptase reaction of 1 hour (lane 5) was compared to 2 h (lane 6); 10 units of reverse transcriptase (lane 6) were compared to 20 units (lane 7). In all cases the quality and quantity of the extension products was the same.

Expression of herpes simplex virus thymidine kinase gene.

Expression of HSV TK gene in Xenopus oocytes was assayed by primer extension using a TK specific primer. 7 ng of pTK plasmid were injected into the GV of oocytes and RNA was extracted 24 h later. An analysis of the total RNA with the primer is shown in figure 9 lanes 3 - 6. A major and very prominent doublet between 67 and 76 nucleotides in length was detected; such doublet was not present among the

primer extension products of non injected oocyte RNA (lane 1) and was identified as TK specific. The doublet corresponds to the correctly initiated TK transcripts described by McKnight (personal comunication). In addition to the doublet a series of faint bands of longer size were present: these were not TK since they were also present in the non injected oocyte RNA lane. Thus, the TK specific primer did hybridize to some RNA species in the oocyte in addition to the TK mRNA.

In order to test the reproducibility of the primer extension protocol, duplicate analyses of each sample were performed, and run side by side on a gel (compare lane 3 to lane 4, and lane 5 to lane 6); the results showed that the two lanes in each pair looked alike and that the assay is thus reproducible.

III. Effect of a Nuclear Protein Fraction from Sea Urchin Gastrula on the Expression of Sea Urchin L1H2b Gene; Analysis by Microinjection into Xenopus Oocytes

In previous work, sea urchin gastrula chromatin proteins were fractionated and assayed for the presence of a modulatory activity on the expression of sea urchin LH2b gene. Such an activity was found in a fraction that was a 0.45 M - 1 M NaCl wash of the chromatin; the assay for the activity was the microinjection of protein and gene into Xenopus oocytes and subsequent analysis of the amount of

correct initiation of transcription by S 1 mapping. It was observed that transcription of H2b gene in the presence of this fraction was stimulated. In the results presented here I have further characterized the stimulatory activity of the fraction utilizing the same oocyte microinjection assay coupled to primer extension analysis of the RNA.

The experimental design for these experiments was as follows: first the protein fraction was injected into the cytoplasm of oocytes and these were allowed to incubate for 5 h. The protein fraction could not be coinjected with the plasmid into the GV because it contained nucleases that degraded the DNA before injection. By injecting the protein fraction into the cytoplasm and incubating the oocytes for several hours, it was ensured that nuclear proteins had migrated into the GV (Bonner, 1975 b). DNA was then injected, and oocytes were incubated for another 5 h to allow transcription of the injected gene. At 5 h after injection of a gene transcripts are already detectable (see Figure 5-II) and their accumulation is still a linear function of time.

a) Characterization of the effect of the protein fraction on the transcription of sea urchin LH2b gene.

Dose response of the amount of protein. The effect of increasing amounts of the crude protein fraction on the expression of LH2b was tested (Figure 10). A given amount

of the protein fraction 0 ng (lane 3), 2 ng (lane 4), 5 ng (lane 5), 10 ng lane 6) and 20 ng (lane 7) was injected into the cytoplasm of oocytes and these were incubated for 5 h. Plasmid pSpL1 (4 ng) was injected into the GV of the same oocytes and incubated for 5 more hours. At this point RNA was extracted and analyzed by primer extension using the H2b specific primer.

The results in figure 10 indicate that the protein produces a stimulatory effect on transcription of LH2b gene (compare lane 3 (no protein present) to any of lanes 4 through 7 (2, 5,10 and 20 ng of protein respectively)). The amount of the transcripts increases as the amount of protein increases and is maximal at 10 ng of protein (lane 6).

An estimate of the magnitude of the stimulation was made by scanning the bands of the autoradiographs that corresponded to correct L1H2b transcripts and calculating the ratio transcription in the presence of the protein / transcription of the gene alone in the absence of injected protein. The magnitude of the stimulation produced by 10 ng of protein incubated for 5 h, on the transcription of 4 ng of injected pSpL1 varied between 3 and 30 fold from one experiment to another. The average value from 5 experiments was 10-fold.

At 20 ng of protein (lane 7) there was less stimulation than at 10 ng. It is possible that the RNA in

this sample was partially degraded since some extension bands smaller in size than the band of correct initiation are visible in lane 7 and not in any of the other lanes that contain LH2b mRNA. In another experiment in which 10 and 20 ng of protein were tested for stimulation of transcription of 4 ng of injected pSpL1, 20 ng produced a stronger stimulation than did 10 ng (not shown).

Time course of incubation of the protein fraction in the oocytes. To further characterize the nature of this stimulatory activity, a time course of incubation of the protein was performed. A fixed amount of protein (5ng) was injected into the cytoplasm of oocytes and these were allowed to incubate for increasing amounts of time: 1 h, 2 h, 10 h and 24 h respectively. At these times DNA was injected (4 ng per GV) and oocytes were incubated for 5 h. The results of the primer extension analysis (figure 11) showed that stimulation occurred as soon as 1 h after the injection of the protein (compare lane 4 to lane 3). The results also showed that as the incubation time for the protein increased, the magnitude of stimulation of transcription of the subsequently injected LH2b gene also increased. By 24 h the stimulation was maximal.

b) Studies on the specificity of the stimulatory activity.

One way of testing whether this stimulatory activity is specific for sea urchin histone H2b genes

consists of testing the effect of the same fraction of proteins on the transcription of other genes.

Two genes were chosen for such purpose. The first gene is Herpes simplex virus thymidine kinase gene; this is a viral gene that is not expressed in the sea urchin gastrula. The second gene is a sea urchin gene called Spec1; this gene is expressed at the same developmental stage as is LH2b gene.

The experimental design was the same as for the characterization of the 1 M sakt extracted protein fraction on L1H2b: the protein fraction was injected first, and after 5 h of incubation each gene was injected at the same copy number as was L1H2b. As a positive control, oocytes were injected with pSpL1 with or without the protein fraction. After 5 h RNA was extracted and analyzed by primer extension.

HSV thymidine kinase gene. The thymidine kinase gene was used as the first test gene to study whether the 1 M salt extracted protein fraction stimulates transcription of sea urchin H2b genes specifically. 5 ng of protein were introduced into oocytes and incubation proceeded for 17 h prior to microinjection of 1.7 ng of pTK plasmid into the GV. 1.7 ng of pTK plasmid and 4 ng of pSpL1 contain an equivalent number of copies of the respective genes. 17 h after the DNA injections RNA was extracted and analyzed by primer extension using a TK specific end labeled primer

(figure 12). As a positive control, LH2b gene was injected in parallel, with or without 5 ng of protein respectively (Figure 12 B).

The results in figure 12 indicate that after the 17 h incubation period, the 1 M salt extracted protein fraction did not affect the level of transcripts of TK significantly (compare lanes 2 and 3 in Figure 14 A); in contrast, the level of LH2b transcripts was clearly stimulated (compare lanes 4 and 5 of Figure 12 B).

Spec1 gene. To test whether the 1 M salt extracted protein fraction had a stimulatory effect on the transcription of sea urchin Spec1 gene, the fraction was microinjected into the cytoplasm of Xenopus oocytes and these were incubated for 5 hours. 2 ng of pSpec1.1 were injected into the GV and injected oocytes were incubated for 5 h. RNA was then extracted and analyzed by primer extension with an end labeled Spec 1 specific primer. A dose response of the amount of protein was performed by injecting increasing amounts of protein (2 ng, 5 ng, 10 ng, and 20 ng) in different groups of oocytes. As an internal positive control, other oocytes were injected with 10 ng of the protein fraction and 4 ng of the sea urchin LH2b gene.

The dose response of the 1 M salt extracted protein fraction on the transcription of Spec1 gene is in figure 13 A. In all lanes the amounts of transcription were similar and a stimulation comparable to that observed

for LH2b was not observed (compare Figure 13 A to figs. 13 B and 10). Spec1 mRNA specific signals were slightly darker in the lanes that contained the samples corresponding to injection of 2 ng or 5 ng of protein; the signals were slightly fainter for the samples corresponding to injection of 10 ng or 20 ng of protein. Because the intensity of the bands is low, and because the variation among the different lanes is small it is not possible to determine whether the variation observed represents a real trend or if it merely reflects the inherent random variation of the injection experiments.

Figure 13 B shows the positive control for Spec 1 experiment. Transcription of 4 ng of injected LH2b gene in the presence of 10 ng of protein is stimulated 40 fold above the basal level.

Another dose response of the 1 M salt extracted protein fraction on transcription of Spec1 gene was performed (not shown), and the results are consistent with those shown in figure 13; they again indicate lack of stimulation and overall low transcription levels. LH2b controls run in parallel showed approximately 5 fold stimulation at 10 ng of protein. It appears that under similar conditions of gene copy number and amount of protein used, transcription of Spec1 genes fails to be stimulated by the 1 M salt extracted protein fraction in the way that LH2b genes are stimulated.

DISCUSSION

I. Parameters of the Oocyte Microinjection System for the Study of Transcription

a) Quantitation of gene expression in oocytes.

Microinjection into Xenopus oocytes has been used as a transcription assay to study the effect of trans-acting molecules on the expression of the sea urchin L1H2b gene. The advantages of this system are that it is an in vivo assay and that it is applicable for the study of a wide variety of genes. The limitation of the system is in its intrinsic variability. One source of variability is in the microinjection of DNA into the GV. I assayed my accuracy of delivery of DNA into the GV and it was 88 % on the average. The other source of variability is in the efficiency of transcription among different batches of oocytes from one female and among oocytes from different females. This accounts for disparate levels of transcripts between replica experiments performed using different batches of oocytes. For this reason it is difficult to measure absolute values of transcription levels. The information obtained from microinjection experiments reveals trends and qualities of transcription. In order to quantitate levels of transcription it is necessary to average large numbers of replica experiments and obtain estimate values.

b) Enzyme activity is an indirect evidence of transcription.

The study of transcription involves the detection and analysis of RNA transcripts. In the oocyte these transcripts get translated into protein and in the cases where the protein is a known enzyme, it is possible to assay for enzyme activity. Such is the case for CAT. Eukaryotic promoter sequences have been cloned upstream of the CAT gene and their function has been tested by analyses of CAT enzyme activity. Enzyme activity is an indirect evidence that transcription has occurred. The levels of enzyme activity nevertheless, do not necessarily correlate with those of correct RNA transcripts since translation is a separate process form transcription that has its own rate limiting steps.

Expression of CAT injected into oocytes has been analyzed at the transcriptional and translational levels. The time course of appearance of CAT enzyme activity shows that it appears between 10 and 24 h after injection. On the other hand appearance of CAT transcripts is detected as soon as 2.5h after injection. There is also a lag time between transcription and translation. A similar lag of time between appearance of transcripts and appearance of protein has been observed after injection of the adenosine deaminase (ADA) gene (Bigo, personal communication). ADA transcripts appeared 2 h after injection whereas ADA

enzyme activity was not dected until 15 h after injection. Translation of injected mRNAs however, is known to occur as soon as 2 h. after injection (Colman and Morser, 1979). This time may be required for the maturation of primary transcripts and the translocation of the mature mRNAs to the cytoplasm. Alternatively, only a small fraction of the messages are viable for translation. In this respect I have shown that transcription of CAT gene from pSV2CAT plasmid occurs from several aberrant start sites (Figure 7). These aberrant messages may be detected early after injection but may fail to be properly processed and translated (Probst et al. 1979).

c) Genes differ in their efficiency and fidelity of transcription in the oocyte.

I have studied the transcription of cloned genes injected into Xenopus oocytes using the technique of primer extension. The analysis of four different genes showed that their patterns of expression in the oocyte were different.

Fidelity of transcription (as defined by a preferred utilization of the correct start site) was observed for L1H2b gene, Spec gene and HSV tk gene but was not observed for CAT. Correct initiation of L1H2b gene had been previously observed by Maxson et al. (86), and correct initiation of HSV tk was reported by McKnight et al. (81). In this paper I report that the Spec 1 gene promoter is

also properly recognized by the oocyte's transcriptional machinery.

The primer extension analysis of CAT transcripts (Figure 7) showed that these started at multiple sites on the plasmid and that initiation at the proper start site was not preferred over the aberrant sites. Three of the aberrant start sites were whithin the SV40 promoter region and one site was within the pBR322 vector. Aberrant initiation within SV40 sequences probably reflects an improper regulation of the SV40 early promoter by the oocyte's transcription machinery. Aberrant initiation of transcription of genes injected in oocytes has also been reported for the ovalbumin gene, rabbit b-globin gene, and sea urchin H4 gene (reviewed by Gurdon and Melton, 81). Transcription of prokaryotic sequences from a plasmid vector in a eukaryotic transcription system has also been reported in the transformed yeast cells (Marczinski and Jaehning, 85).

The efficiency of transcription (as defined by the absolute levels of correctly initiated transcripts) was low for L1H2b, Spec and CAT genes as compared with HSV tk gene. In the case of the HSV tk gene, the high level of transcriptional fidelity agrees well with what has been previously reported (McKnight et al. 1981). It has been suggested that the high efficiency and fidelity of transcription of HSV tk in the oocyte reflects the fact that

virus genes are designed to function in a variety of host cells (Gurdon and Melton, 81). In the same line of thought it has been suggested that genes whose normal expression is cell-type specific show improper transcription in the oocyte. If that were true one whould expect high levels of correct CAT transcription from the SV40 early promoter in pSV2CAT and this was not the case. The improper regulation of pSV2CAT transcription as well as the high levels of HSV tk transcription in the oocyte are interesting questions that require further investigation.

II. Effect of a SeaUrchin Gastrula Protein Fraction on the Transcription of Sea Urchin Late Histone Genes

a) Description of the stimulatory activity.

I have characterized the stimulatory effect of a fraction of proteins from sea urchin gastrula chromatin on the transcription of sea urchin histone L1H2b gene injected into Xenopus oocytes. I showed how the levels of correctly initiated transcripts increased in the presence of increasing amounts of the protein fraction (see Figure 10). Levels of L1H2b mRNA increased linearly until 10 ng of the fraction were injected. Maximum stimulation was 10 fold on average with a range of 3 to 30 fold. Something in the fraction is clearly stimulating transcription. Since the protein fraction used contains a mixture of proteins, it is possible that the stimulation observed is effected by one or several protein factors. To investigate this question

the protein extract needs to be further fractionated.

When quantities greater than 10 ng of the protein were injected the levels of transcripts increased in one instance and decreased in another instance (not shown). The stimulatory activity may plateau between 10 and 20 ng of the protein fraction. It is possible that with 20 ng of extract inhibitory molecules are present at high enough levels that their activities counteract the stimulation. A purer fraction and an in vitro transcription assay may provide more controlled means to study the kinetics of this activity and to discern between these possibilities.

When the protein fraction was allowed to incubate in the oocyte for increasing amounts of time before the injection of the DNA, it was found that the longer the pre-incubation of the protein, the more transcription was obtained in the 5 h period in which the genes were allowed to transcribe. This result suggests that maximal translocation of the effector molecule from the cytoplasm to the nucleus takes between 10 and 24 h. This would agree with Bonner (75 a). He has shown that the time required for proteins to enter the GV depends on their size. Small proteins 18 x 10^3 daltons or less equilibrate in 24 hours or less, while larger proteins enter the GV more slowly. A question that remains unanswered is whether the activity needs more than 24 h to be fully active.

Another possibility would be that the effector

molecule needs to undergo some chemical modification in order to become active. A purer fraction will permit the coinjection of proteins and DNA into the GV and it will be possible to see whether the activity appears at time zero or alternatively, if it needs time for a chemical modification step.

b) The stimulation of transcription is specific for L1H2b.

It has been shown that a fraction of chromatin proteins from sea urchin gastrula is able to stimulate transcription of sea urchin L1H2b genes. The fraction may contain a transcription factor that is specific for the regulation of L1H2b genes. Alternatively, the activity may be general and stimulate transcription of any pol II gene. I have approached this question by testing the effect of the protein fraction on the transcription of other genes. First, I studied the HSV tk gene. As shown in Figure 12, 5 ng of the protein fraction failed to stimulate transcription of the HSV tk gene while the same amount of protein fraction clearly stimulated transcription of L1H2b gene.

It appears that the stimulatory activity does not affect HSV tk transcription in the same way that it affects L1H2b. One possible reason for this is that the basal rate of transcription of tk is already maximal and thus, the factor is unable to rise it further. Alternatively, the activity may be totally unrelated to the transcription of

tk gene.

To further investigate whether the activity is genuinely specific for L1H2b, I next tested the effect of the fraction on the Spec 1 gene. This gene is expressed at the same developmental stage as the L1H2b gene in the sea urchin. Also, its basal level of transcription in the Xenopus oocyte is similar to that of the L1H2b gene.

As shown in Figure 13 the levels of Spec 1 transcripts synthesized in the presence of increasing amounts of the protein fraction do not increase significantly above the level of transcripts synthesized in its absence. On the other hand, the level of transcripts of the L1H2b gene is clearly higher in the presence of the protein fraction.

The presence of a crude mixture of sea urchin gastrula chromatin proteins in the oocyte correlates with an increased amount of transcription of L1H2b but does not correlate with increased transcription from HSV tk or Spec 1 genes. Because transcription of HSV tk and Spec 1 genes failed to be stimulated by the protein fraction the results indicate that the activity observed is not general. Instead, the stimulatory activity seems to affect the transcription of L1H2b specifically.

c) Specificity implies recognition of L1H2b DNA sequences.

The fact that the activity is specific implies that some molecule in the protein fraction recognizes some specific sequence on the pSpL1 plasmid. Experiments have been done to test whether any molecule in the protein fraction is able to bind to specific DNA sequences in the L1H2b gene. Gel electrophoresis DNA binding assays (Fried and Crothers, 1981) revealed that a BamHI restriction fragment (Bam 105) of L1H2b DNA binds specifically to some molecule of the protein fraction. This fragment spans over 61 bp of leader sequences of the L1H2b gene and 44 bp of upstream sequences that contain the TATA box.

DNA binding by molecules from a crude protein fraction does not necessarily imply that these components have a modulatory function on gene transcription. It is necessary to prove that there is correlation between binding and stimulation of transcription. In this respect, competition experiments have been performed injecting increasing amounts of Bam 105 fragment along with the L1H2b gene into oocytes and testing if the protein fraction was able to stimulate transcription of the latter Maxson et al. 1986). If binding and transcription stimulation are effected by the same molecule then, increasing amounts of Bam 100 fragment will sequester it and stimulation of L1H2b genes will be competed away. The results showed that

indeed, when the molar proportion of L1H2b gene to Bam 105 fragment was 1:5 there was no stimulation of transcription of L1H2b.

The results of the binding experiment and the competition experiment agree with the notion that the protein fraction contains a stimulatory activity specific for the transcription of L1H2b. A more thorough assessment of the sequences involved in recognizing the effector molecule(s) needs to be done by testing the responsiveness of deletion mutants of the L1H2b gene to the protein fraction. Eventually, footprints of the protein bound to DNA will reveal the exact binding site. This experiment awaits the availability of a purer fraction.

d) Experiments for the future.

To standarize the results of the magnitude of stimulation it is necessary to optimize the system further. The time course of protein incubation needs to be extended to find the time at which migration of the protein (or its activation) is completed. This time should represent an optimized parameter. To correct for the inherent variability among batches of oocytes, experiments should be repeated enough times that statistics can be applied and the standard deviation of the values is reduced.

Further questions that need to be investigated include 1) whether the activity is mediated by one or more molecules; 2) what are the sequences of DNA involved in

the recognition of these molecules; and 3) whether the activity affects initiation or elongation of transcription.

These shall be approached by pursuing the purification of the activity as well as generating deletion mutants of the L1H2b DNA sequences and testing their ability to be stimulated.

A purer fraction of proteins and the identification of the sequences involved in mediating the activity will allow finer kinetic studies as well as the possibility of doing footprinting experiments to investigate the mode of action of the effector molecules. The development of an in vitro transcription system may be necessary to study the mechanics of the stimulatory activity.

III. Significance of This Work

a) Regulation of genes along development.

Early and late sea urchin histone genes have different patterns of expression in development. In this work I have shown that a fraction of gastrula proteins stimulates transcription of late genes specifically. Mous et al. (1985), have described another chromatin protein fraction that stimulated transcription of sea urchin histone H2b genes injected into Xenopus oocytes. They studied the early H2b gene of a different species of sea urchin (Psammechinus militaris) and their chromatin protein fraction was prepared from an earlier developmental stage than gastrula (64 - 128 cell morulae of Paracentrotus

lividus). The active fraction was found in a .3 - .45 M NaCl wash of chromatin. At this point, the data is not sufficient to decide whether the activity described here and the activity described by Mous et al. are the same or not.

In previous work (Maxson et al. 86) we showed that early histone genes are also stimulated by this fraction but to a lesser extent. Competition experiments show that early and late gene stimulation is effected by the same factor (not shown). It would be interesting to see whether this factor is involved in the developmental regulation of this gene pair.

The pattern of expression of early and late histone genes is reminiscent of that observed for the oocyte and somatic types of 5 S RNA genes in Xenopus (Brown, 1984). As described in the introduction, the oocyte type genes are only active during oogenesis and repressed during embryogenesis, while the smaller set of somatic genes continues to be expressed. Somatic type genes appear to have a higher affinity for the transcription factor TFIIIA than the oocyte type genes.

A similar explanation may be applicable for the differential transcription of the sea urchin early and late histone genes.

In the sea urchin, the early genes are abundant and are expressed early in development while the few copies

of the late genes continue to be expressed beyond gastrula stage. It would be interesting to investigate whether the amount of the factor(s) involved in stimulating transcription of the H2b genes (per cell) is higher in early sea urchin development than at gastrula. This will be possible once this factor(s) is purified.

It would also be interesting to determine if the observed stimulatory activity for the transcription of the late H2b genes is involved in the regulation of histone synthesis during the cell cycle.

TABLE I

Characteristics of the Recombinant Plasmids

Name	pSV2CAT	pSpL1	ptk /. \3'-1.13/ \5'-182	pSpec 1-1
Gene	a) SV40 early promoter + enhancer b) Chloramphenicol acetyl-transferase (CAT)	late 1 histone H2b (L1H2b)	thymidine kinase (tk)	S.purpuratus ectoderm 1 gene/ 1 exon (Spec 1-1)
Species	a) Simian virus 40 b) E. Coli	Sea urchin S.purpuratus	Herpes simplex virus (HSV)	Sea urchin S. purpuratus
Insert size (*)				
5'	.34	3.0	.182	2.7
coding region	.77	.5	1.3	.22 1st exon 1.38 portion
3'	1.6	6.0	.294	1st intron
total	2.7	9.5	1.8	4.3
Vector				
origin	pBR322	pBR322	pBR322	pUC8
size (*)	2.3	4.3	4.0	2.7
cloning site	PvuII/EcoRI	EcoRI/HindIII	BamHI/HindIII	SalI
Total size (*)	5	13.8	5.8	7
Reference	Gorman et al. (1982)	Maxson et al. (1983)	McKnight et al.(1981)	Hardin et al. (1985)

(*) : in Kilobases

TABLE II
Characteristics of the oligonucleotide primers

Gene specificity	Size of primer(*)	Sequence	Size of extended product(*)	Td(°C) (**)
sea urchin H2b gene	17	5' TTGTA GATGT AGATT CC 3' [1]		46
early			205	
late			150	
CAT gene	20	5' CAACG GTGGT ATATC CAGTG 3' [2]	123	60
HSV tk	24	5' GGGAG TTTCA CGCCA CCAAG ATCT 3' [3]	74	74
sea urchin Spec1 gene	15	5' GCACG TGACC ATACG 3' [4]	55	48

(*) in nucleotides
(**) Td = 4(G + C) + 2(A + T); see Materials and Methods
1 - Maxson et al. (1986)
2 - Walker et al. (1983)
3 - McKnight (unpublished)
4 - Hardin et al. (1985)

TABLE III

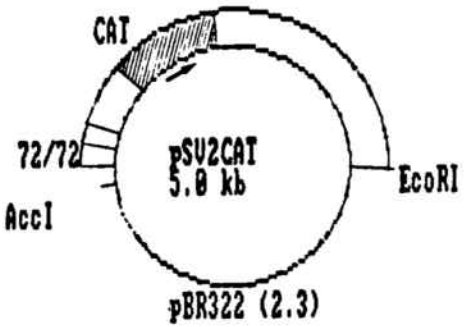
Values of CAT enzyme activity for the samples in figure 5-1

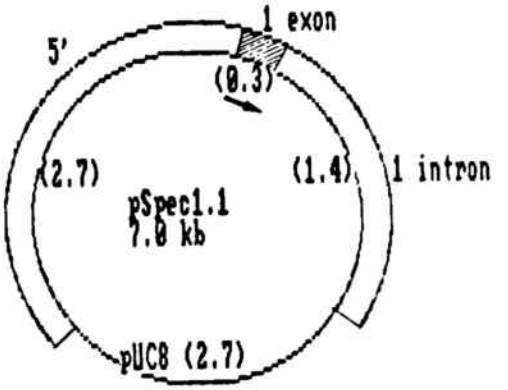
sample	indentification	enzyme activity (*)
A - standard curve		
1	0 u CAT	0.2
2	0.005 u	0.2
3	0.05 u	1.6
4	0.5 u	41.0
5	0.5 u + ni(**)	40.0
B - dose response		
3	0.3 ng pSV2CAT	1.16
2	0.6 ng	13.53
4C	0.9 ng	32.98
1	1.5 ng	81.89
C - time course		
1	2.5 h	< 0.2
2	5.0 h	"
3	10.0 h	"
4	24.0 h	32.98

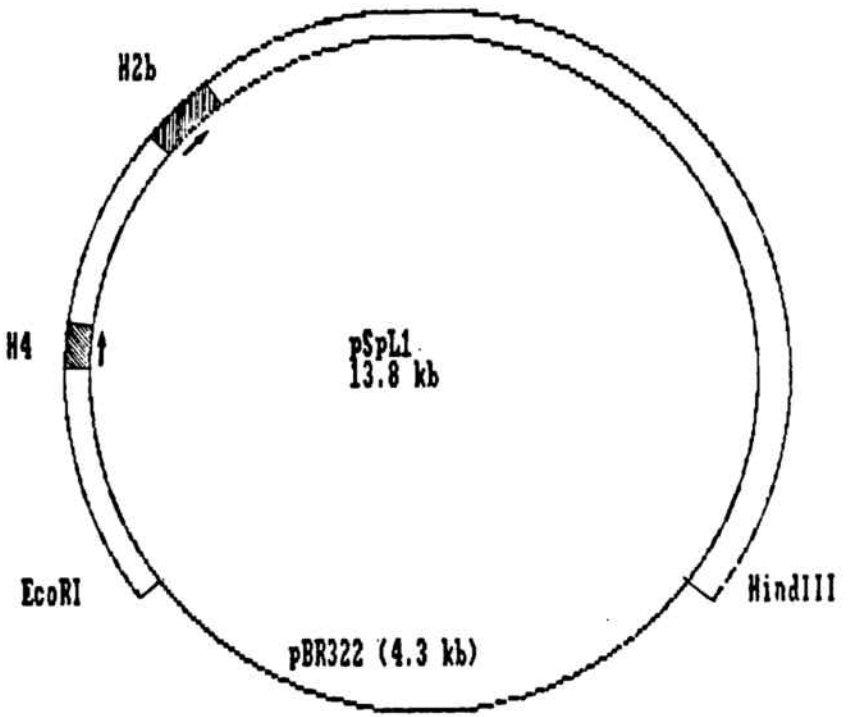
(*) % conversion of nonacetylated chloramphenicol to the acetylated form.

(**)see legend to figure 5-1 A, lane 4.

Figure 1: Diagrams of the recombinant plasmids


CAT
72/72
AccI
pSV2CAT
5.0 kb
EcoRI
pBR322 (2.3)


1 exon
5'
(0.3)
(2.7)
(1.4)
1 intron
pSpec1.1
7.0 kb
pUC8 (2.7)


H2b
H4
pSpL1
13.8 kb
EcoRI
HindIII
pBR322 (4.3 kb)

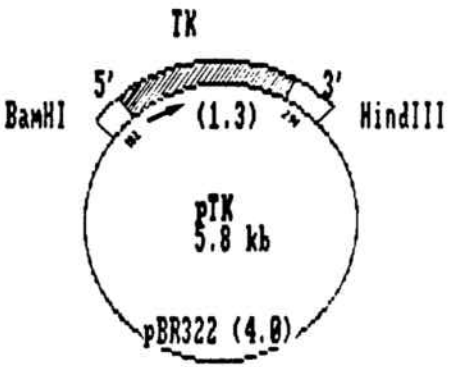
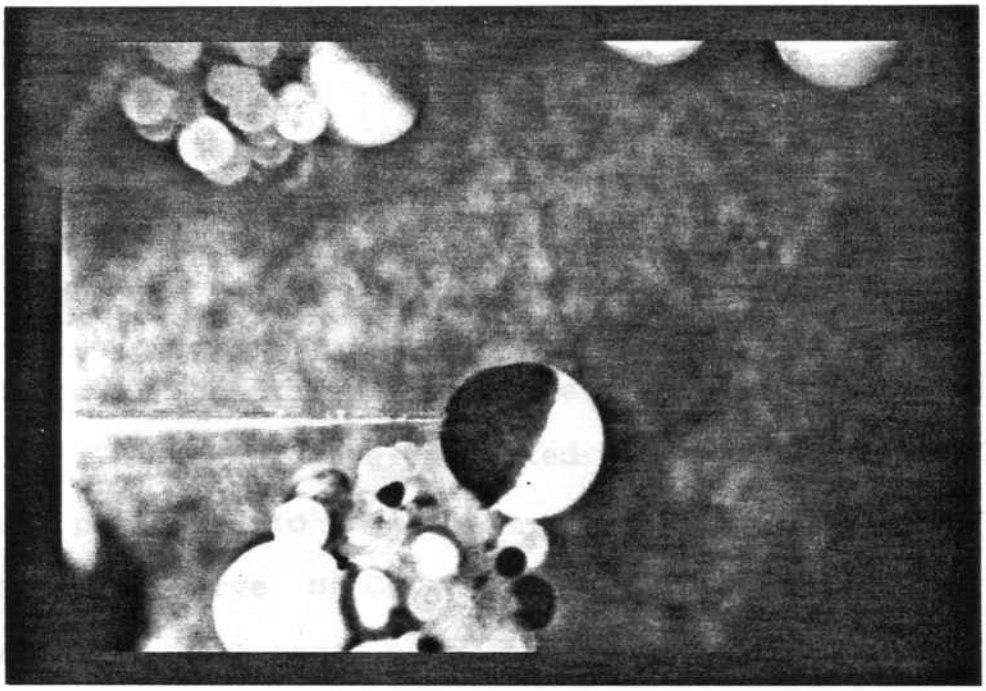

TK
5'
3'
BamHI
(1.3)
HindIII
pTK
5.8 kb
pBR322 (4.0)

Figure 2: Landmarks of the Xenopus oocyte for nuclear and cytoplasmic injections.

Animal Pole



Equatorial Region

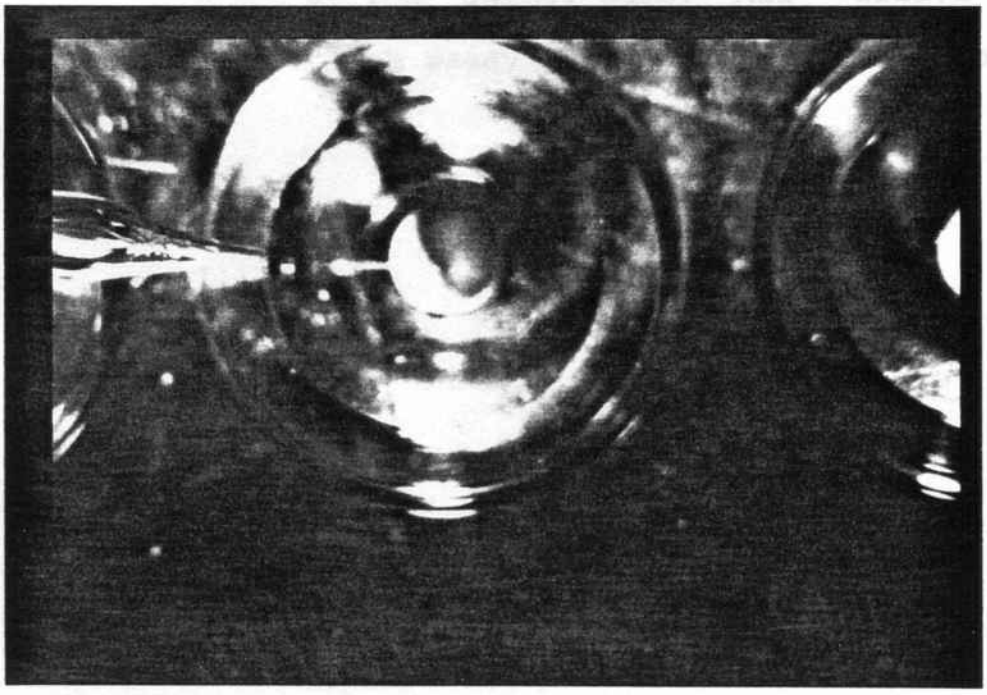
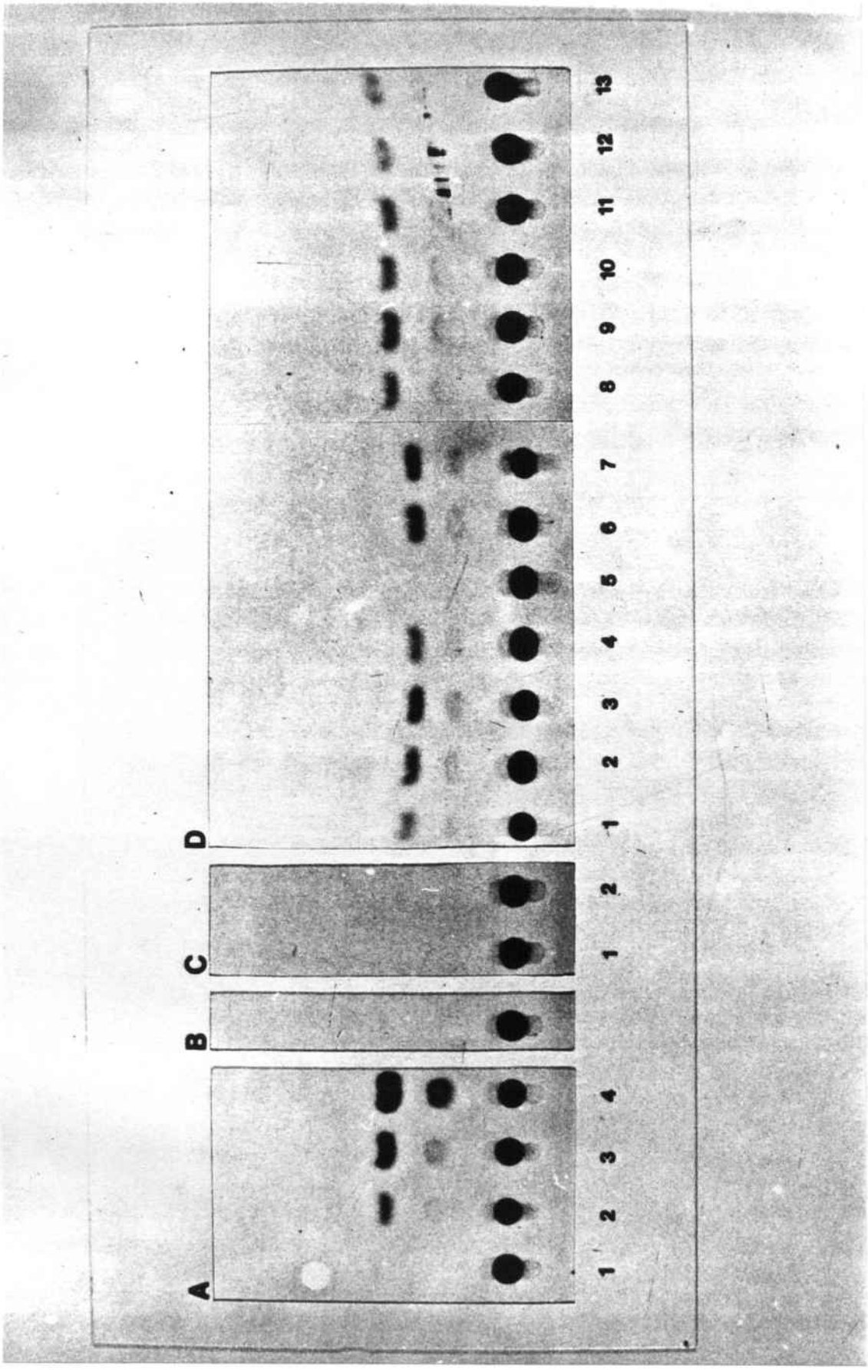


Figure 3: Determination of the accuracy of microinjection. Oocytes were injected with pSV2CAT and CAT assays were performed to assess the accuracy of microinjection. A: Standard curve of CAT activity with 0, 0.05, 0.1 and 0.5 units. B: CAT assay on a non injected oocyte. C: Assays on oocytes injected in the equatorial region. D: Assays on oocytes injected at the animal pole. All assays were on single oocytes. Each assay contained 1/ 10 of an oocyte's equivalent.


A
1 2 3 4
B
C
1 2
D
1 2 3 4 5 6 7 8 9 10 11 12 13

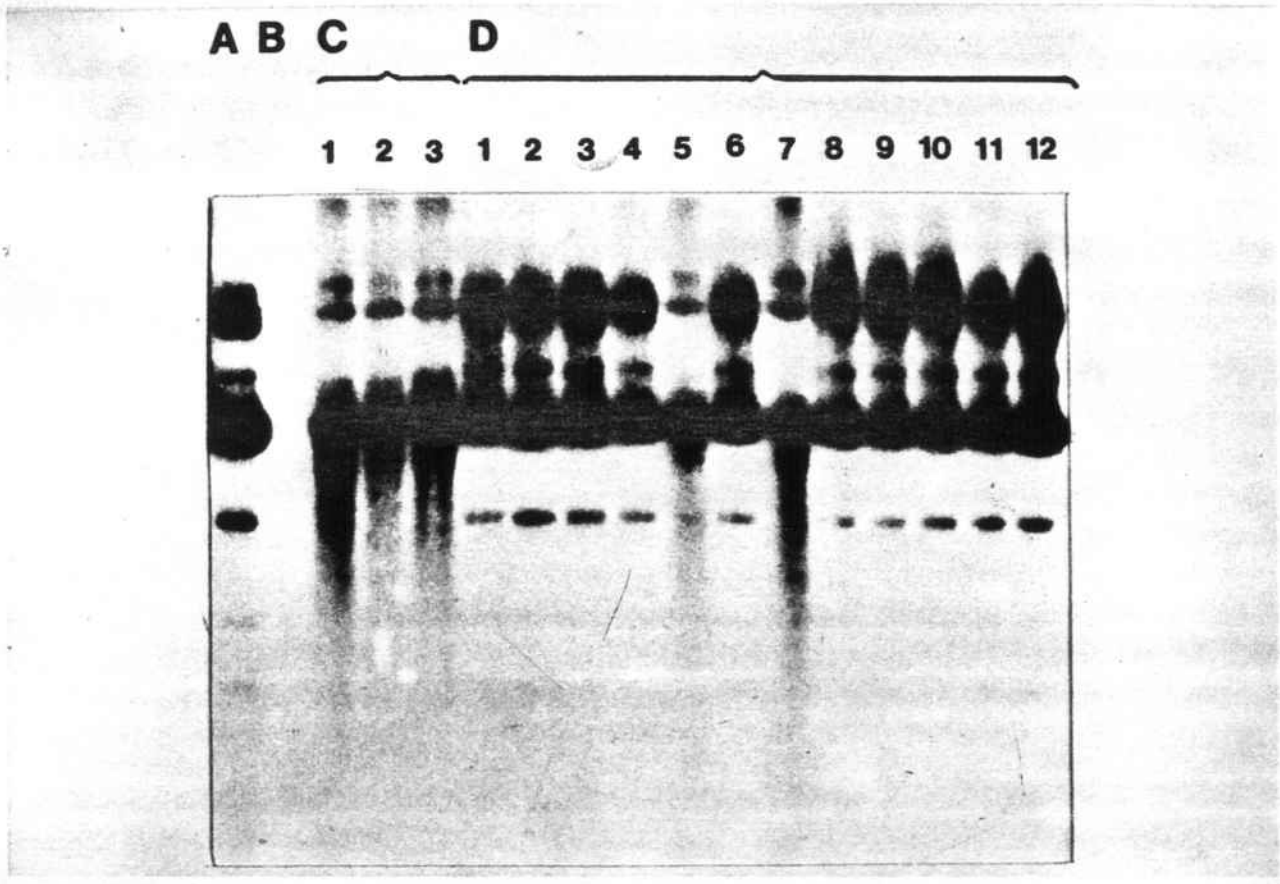


Figure 4: Determination of the integrity of injected DNA.

Oocytes were injected with 1.6 ng of pSV2CAT either at the equatorial zone (C), or at the animal pole (D). DNA from single oocytes was extracted and electrophoresed on an agarose gel; then it was blotted onto a nylon membrane and hybridized to a nick-translated probe of pSV2CAT. A: control plasmid: 1.6 ng of pSV2CAT; B: non injected oocyte DNA.

Figure 5: Expression of pSV2CAT injected into oocytes.
I - Analysis at the protein level by CAT assays.

A: Standard curve of CAT enzyme: 0 u (1), 0.005 u (2), 0.05 u (3), 0.5 u (4) and 0.5 u in an homogenate of a non injected oocyte.

B: Dose response of the amount of pSV2CAT injected: oocytes were injected with 1.5 ng (1), 0.6 ng (2) or 0.3 ng (3) of pSV2CAT and incubated for 24 h before the assays.

C: Time course: oocytes were injected with 0.87 ng of pSV2CAT and incubated for 2.5 h (1), 5.0 h (2), 10 h (3) or 24 h (4) before the assay.
All assays in B and C were done on pools of 5 oocytes
II - Time course of appearence of CAT transcripts in oocytes injected with pSV2CAT.

Oocytes were injected into the GVs with 1 ng of pSV2CAT (B) or 3 ng in (A). RNA was extracted at various times following injection. The RNA was denatured, then spotted onto nitrocellulose. The dot blot was hybridized with a nick-translated probe of pSV2CAT. A: lane 1: RNA from 10 noninjected oocytes; lane 2: RNA from 10 injected oocytes 5 h following injection; lane 3: RNA from 10 injected oocytes 10 h following injection. B: lane 1: RNA from 6 noninjected oocytes; lane 2: RNA from 6 injected oocytes 0 h following injection; lane 3: RNA from 6 injected oocytes 2.5 h following injection; lane 4: RNA

from 6 injected oocytes 5 h following injection.

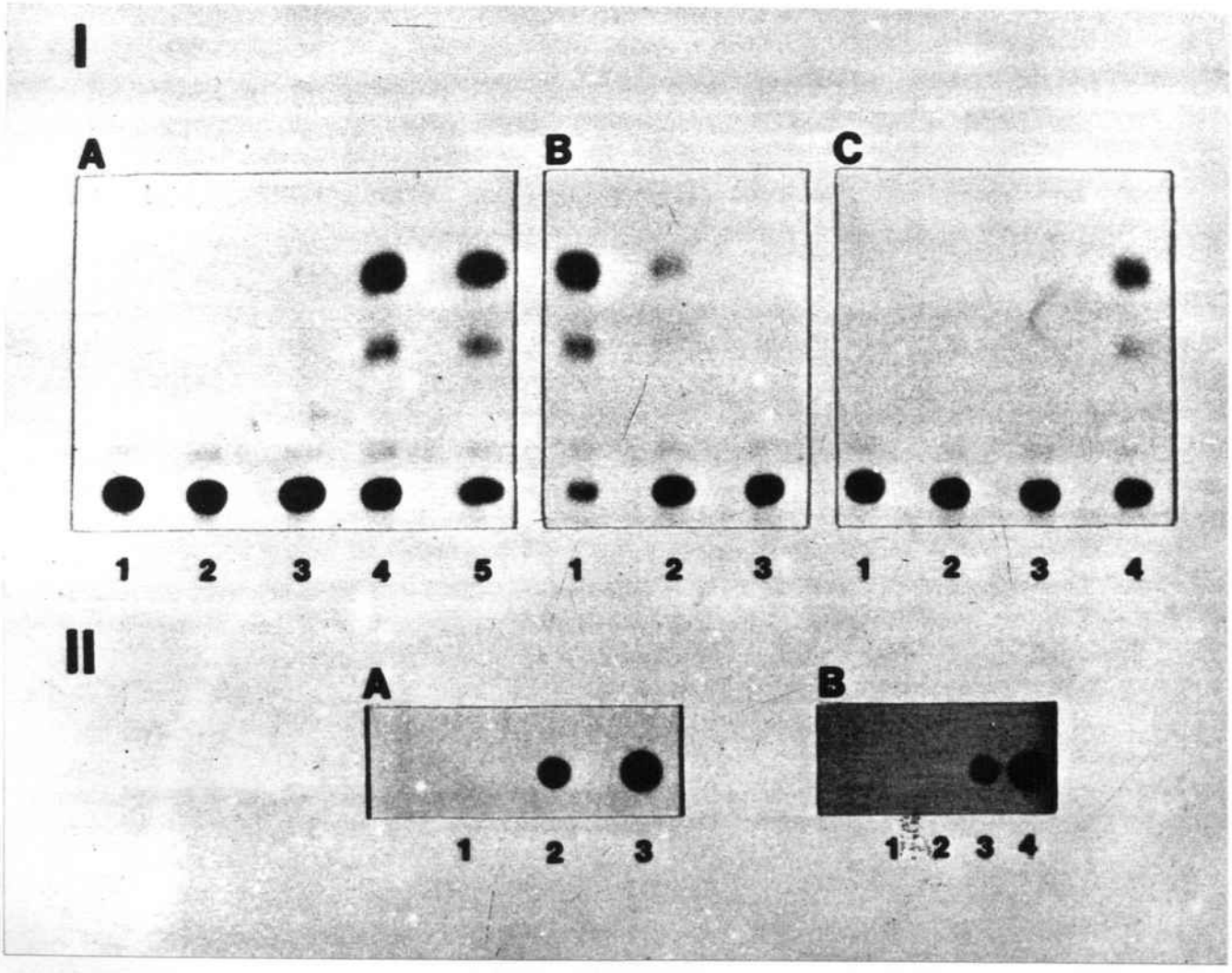
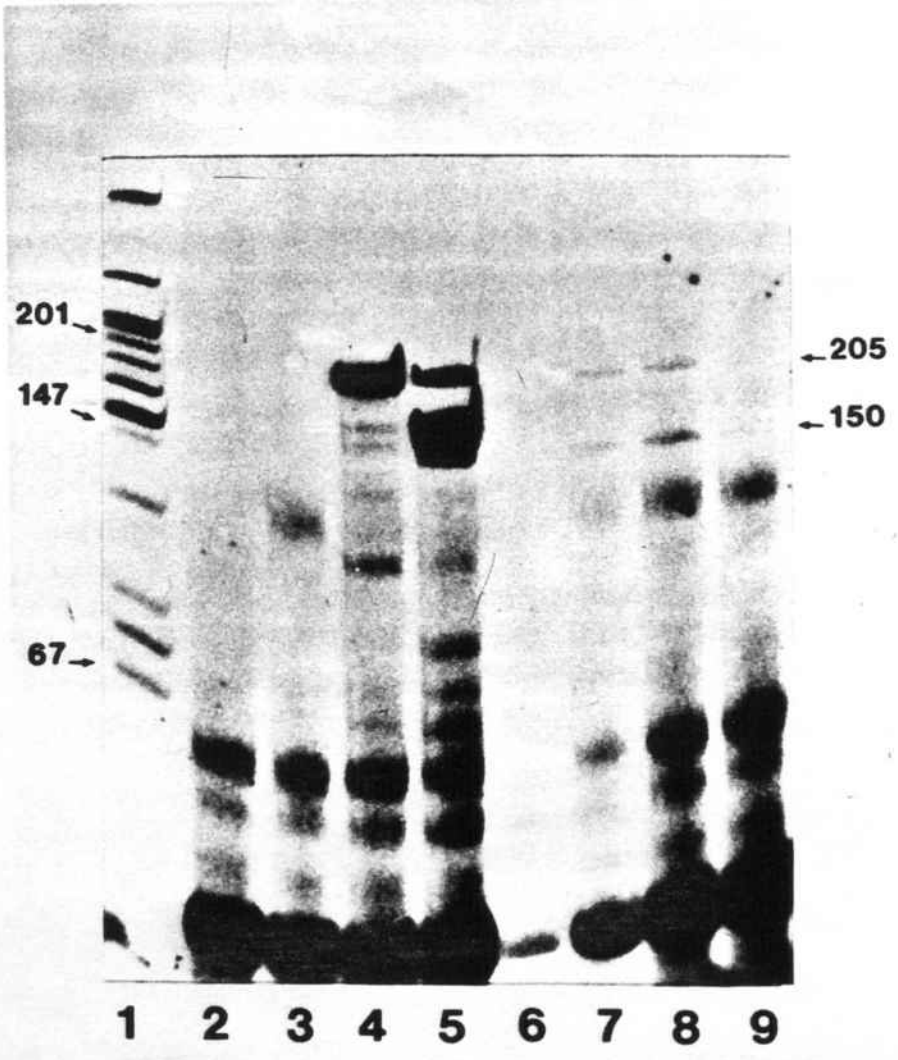


Figure 6: Primer extension analysis of sea urchin LH2b mRNA transcribed in oocytes injected with pSpL1.

Oocytes were microinjected with 4 ng of plasmid pSpL1 and incubated for 24 h before RNA extraction. Primer extension assays were performed with 5 ug of total RNA and 1 ng of LH2b specific end-labelled primer unless otherwise specified. Experiment 1: different amounts of primer were tested with 5 ug of total oocyte RNA: 40 pg (6), 400 pg (7) and 1 ng (8). Experiment 2: different amounts of total oocyte RNA were tested with 1 ng of primer: 5 ug (8) and 1 ug (9). Lane 1: size marker (pBR322 digested with MspI); lane 2: primer extension of 5 ug of tRNA; lane 3: primer extension of 5 ug of noninjected oocyte RNA; lane 4: primer extension of 5 ug of total sea urchin egg RNA; lane 5: primer extension of 5 ug of total sea urchin gastrula RNA.


201
147
67
205
150
1 2 3 4 5 6 7 8 9

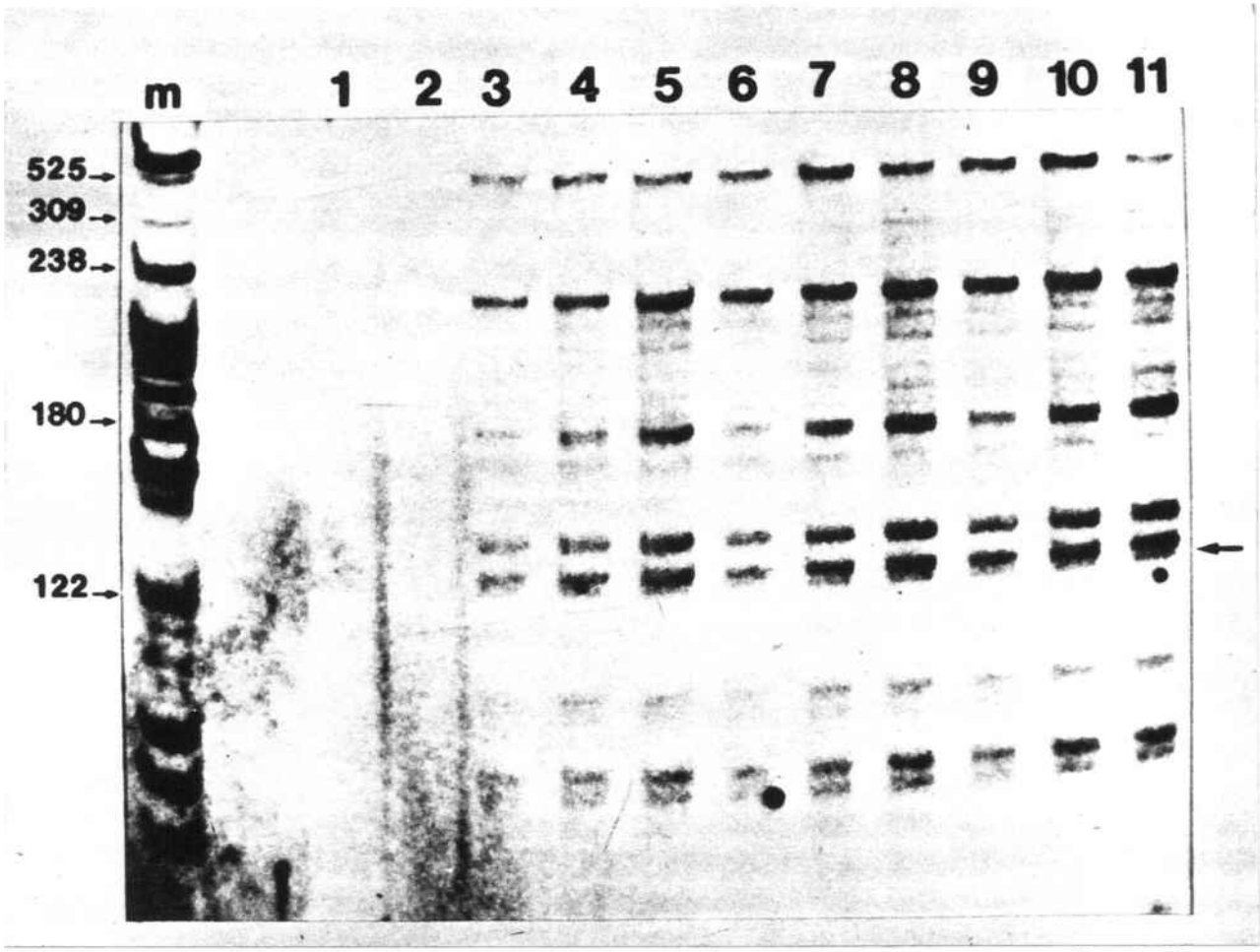
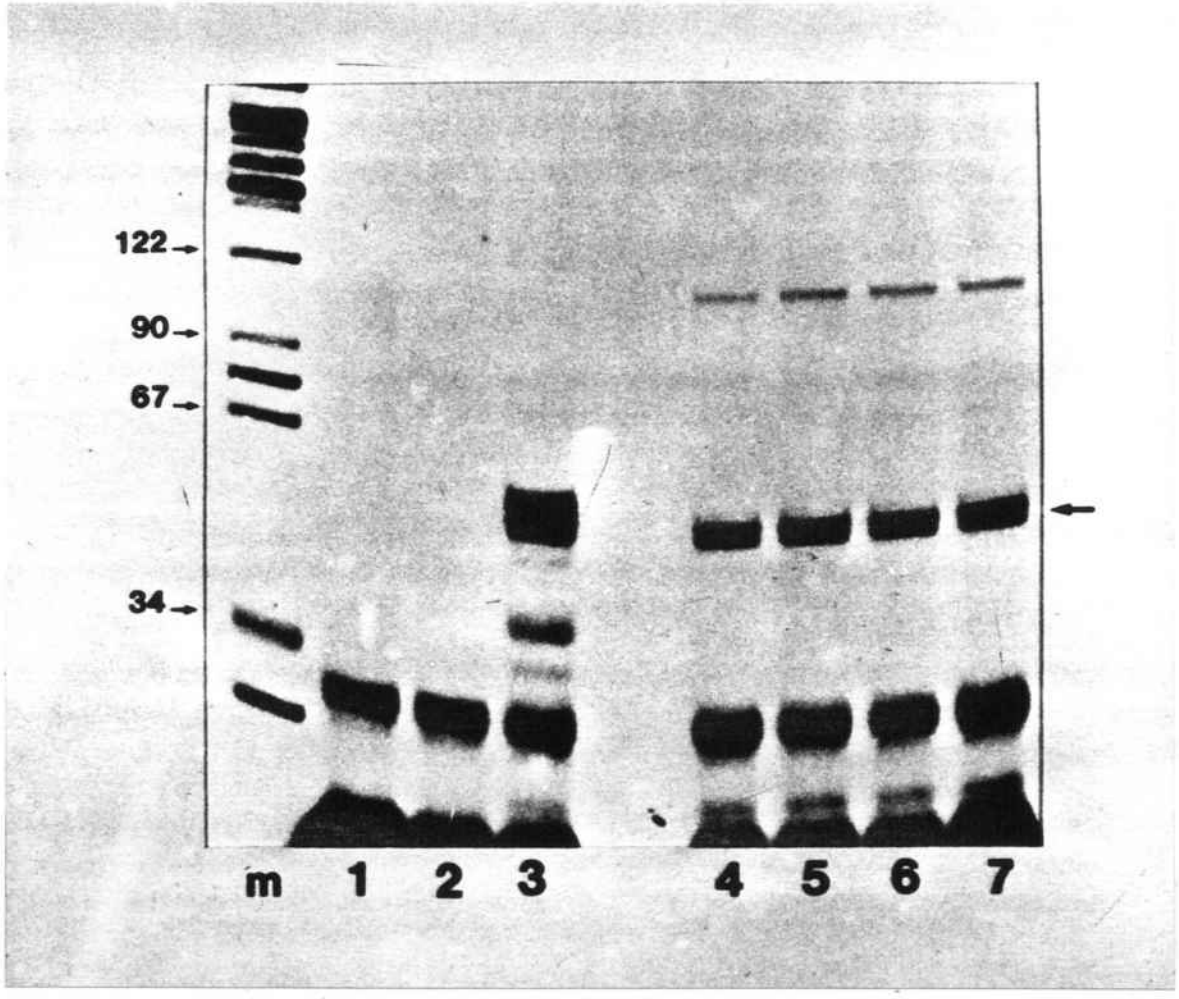


Figure 7: Primer extension of CAT mRNA transcribed in oocytes injected with pSV2CAT.

7 ng of pSV2CAT were injected in oocytes and RNA was extracted 24 h later. All primer extension reactions contained 5 ug of RNA and 1 ng of CAT specific end-labeled primer. Times of hybridization were 1 h (3,4 and 5), 2 h (6,7 and 8) and 4 h (9,10 and 11). Temperatures of hybridization were 45°C (3,6 and9), 50°C (4,7 and 10) and 55°C (5,8 and 11). Lane 1: 5 ug of tRNA; lane 2: 5 ug of RNA from noninjected oocytes. m: size marker. Arrow on the right margin identifies the bands corresponding to the correctly initiated mRNA.

Figure 8: Primer extension of mRNA transcribed from Spec1 gene injected into oocytes.

7 ng of pSpec1-1 were injected in oocytes and RNA was extracted 24 h later. All primer extension reactions contained 5 ug of oocyte RNA and 1 ng of Spec specific end-labeled primer (lanes 4 - 7). Temperatures of hybridization were 38°C in (4) and 43°C in all other lanes. Standard reverse transcriptase reaction parameters were 10 units of enzyme and 1 h of incubation. 20 units of enzyme was tested in (7); 2 h of incubation was tested in (6) and (7). Lane 1: 5 ug of tRNA; lane 2: 5 ug of RNA from noninjected oocytes; lane 3: 10 ug of total sea urchin gastrula RNA. m: size marker. The arrow on the right margin indicates the bands corresponding to the correctly initiated mRNA.


122
90
67
34
m
1
2
3
4
5
6
7

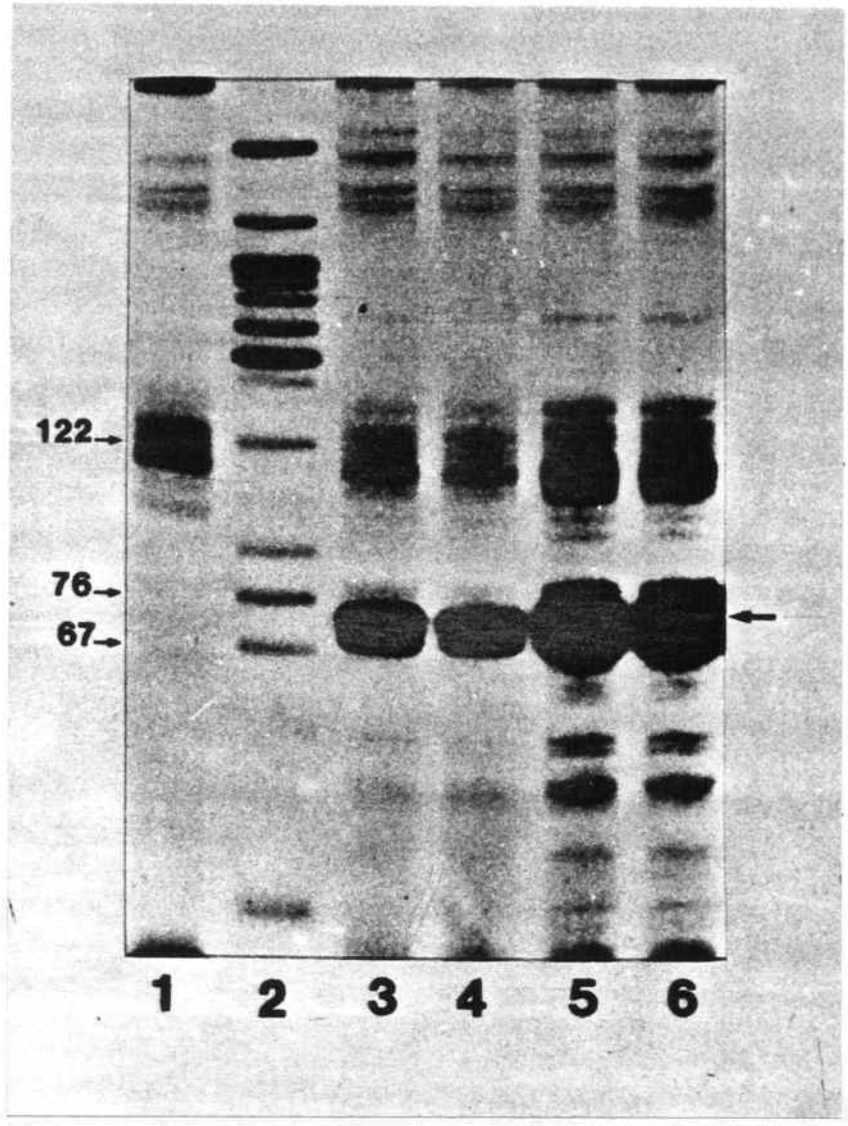


Figure 9: Primer extension of HSV thymidine kinase mRNA transcribed in oocytes injected with ptk.

7 ng of ptk were injected in oocytes and RNA was extracted 24 h later. Primer extension reactions contained 1 ng of TK specific end-labeled primer and varying amounts of RNA as follows: lane 1: 5 ug of RNA from noninjected oocytes; lanes 3 and 4: 0.25 ug of RNA from injected oocytes; lanes 5 and 6: 1 ug of RNA from injected oocytes; lane 2: size marker. The arrow on the right margin indicates the bands corresponding to the correctly initiated mRNA.

Figure 10: Effect of increasing amounts of a sea urchin gastrula protein fraction on the expression of sea urchin LH2b genes injected into Xenopus oocytes.

Details of the experimental design are referred in the text. Amounts of protein injected: 0 ng (3), 2 ng (4), 5 ng (5), 10 ng (6) and 20 ng (7). pSpL1 plasmid was injected at 4 ng / oocyte in all cases. Primer extension reactions containted 5 ug of RNA and 1 ng of primer. Lane 1: 5 ug of sea urchin gastrula RNA; lane 2: 5 ug of RNA from noninjected oocytes; m: size markers are shown on both sides. Arrow: correctly initiated mRNA.

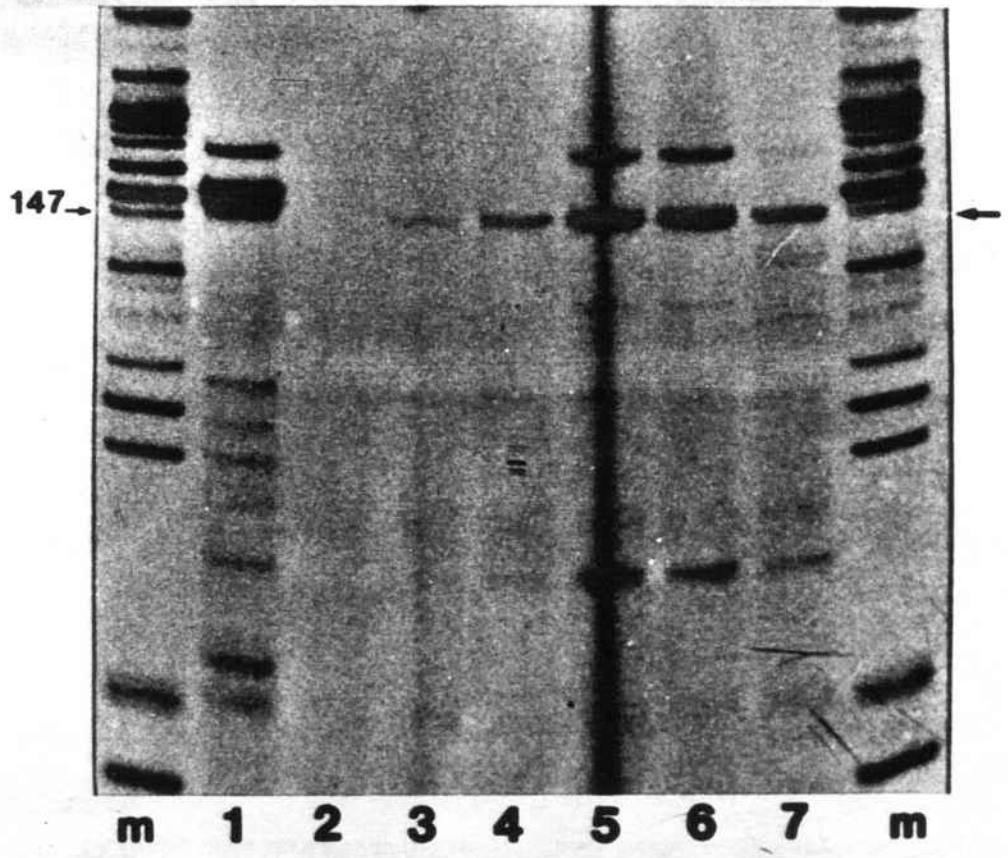

147
m 1 2 3 4 5 6 7 m

Figure 11: Effect of various times of incubation of the protein fraction in the oocyte on the expression of injected L1H2b gene.

5 ng of the protein fraction were injected in the cytoplasm of Xenopus oocytes and these were incubated for 1 h (4), 2 h (5), 10 h (6) or 24 h (7) before the injection of 4 ng of pSpL1. After a second incubation of 5 h RNA was extracted and analyzed by primer extension with an H2b specific end-labeled primer. All primer extension reactions contained 5 ug of RNA and 1 ng of primer. Lane 1: 5 ug of sea urchin gastrula RNA; lane 2: 5ug of RNA from oocytes injected with DNA only; m: size markers. Arrow: correctly initiated mRNA.

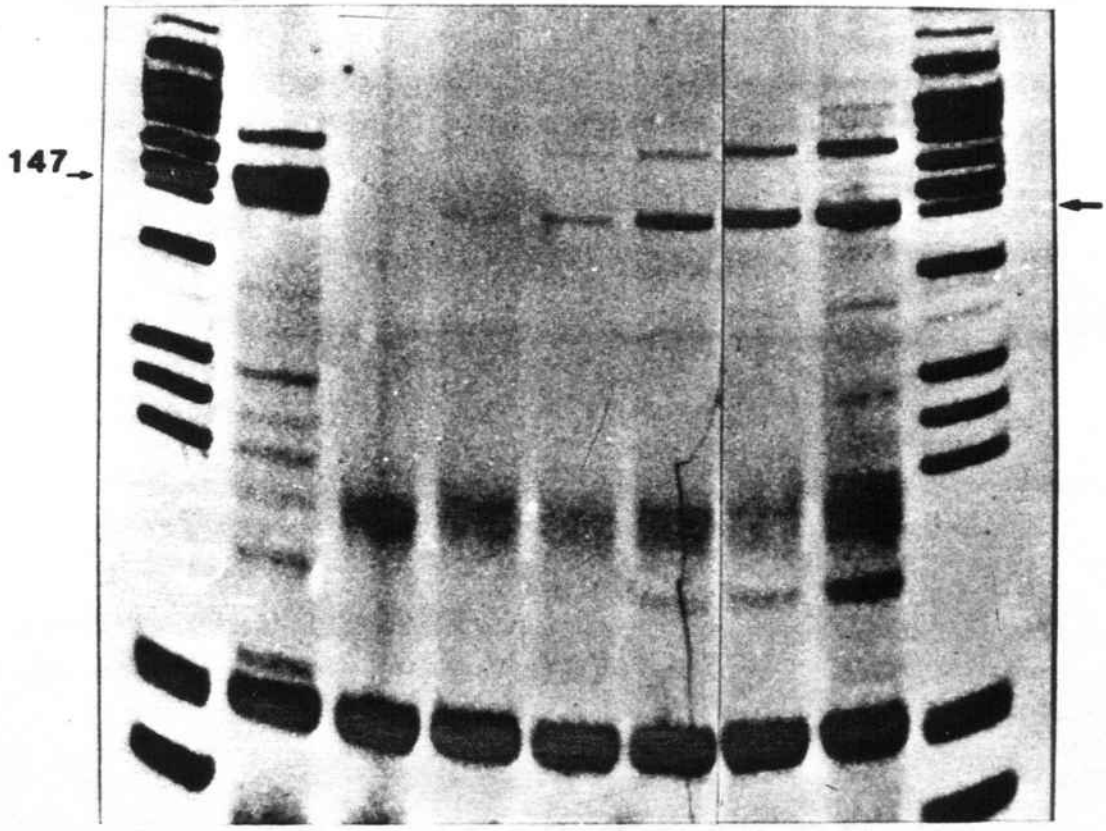

147
m
1
2
3
4
5
6
7
m

Figure 12: Effect of a sea urchin gastrula protein fraction on the transcription of HSV tk gene injected into oocytes.

A: oocytes were injected with 0 ng (lane 2) or 5 ng (lane 3) of protein, incubated for 17 h , injected with 1.7 ng of pTK and incubated for another 5 h prior to extraction of the RNA. Primer extension reactions were as in Figure 14 A. Lane 1: 5 ug of RNA from noninjected oocytes; m: size marker is shown on the left side.

B: as a positive control, the same experiment was repeated injecting 4 ng of the pSpL1 plasmid instead of pTK and utilizing the LH2b specific primer for the primer extension reactions. Lane 1: 1 ug of sea urchin gastrula RNA; lane 2: 5 ug of RNA from noninjected oocytes; lane 3: 0 ng of protein and 4 ng of pSpL1; lane 4: 5 ng of protein and 4 ng of pSpL1.m: size marker.

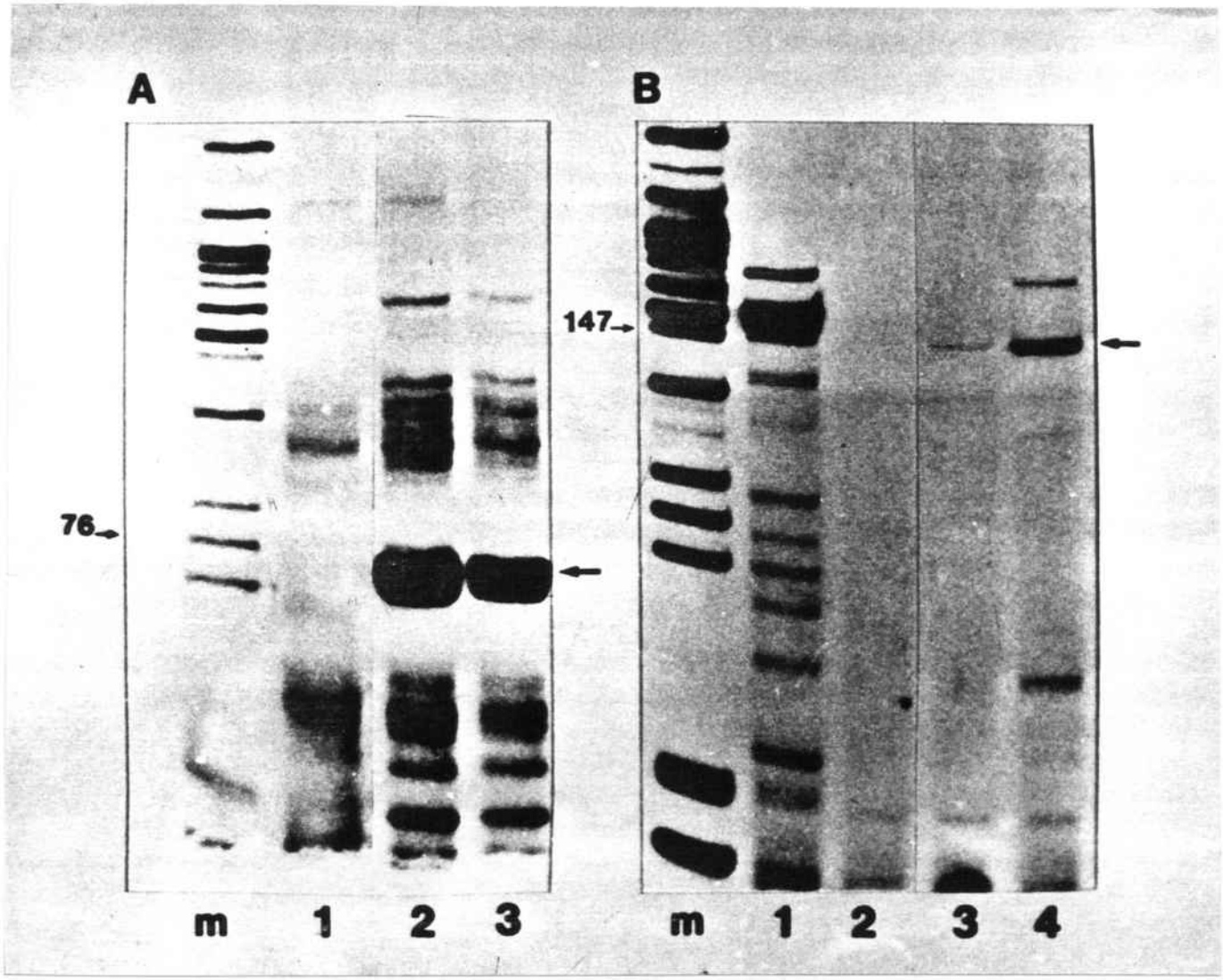
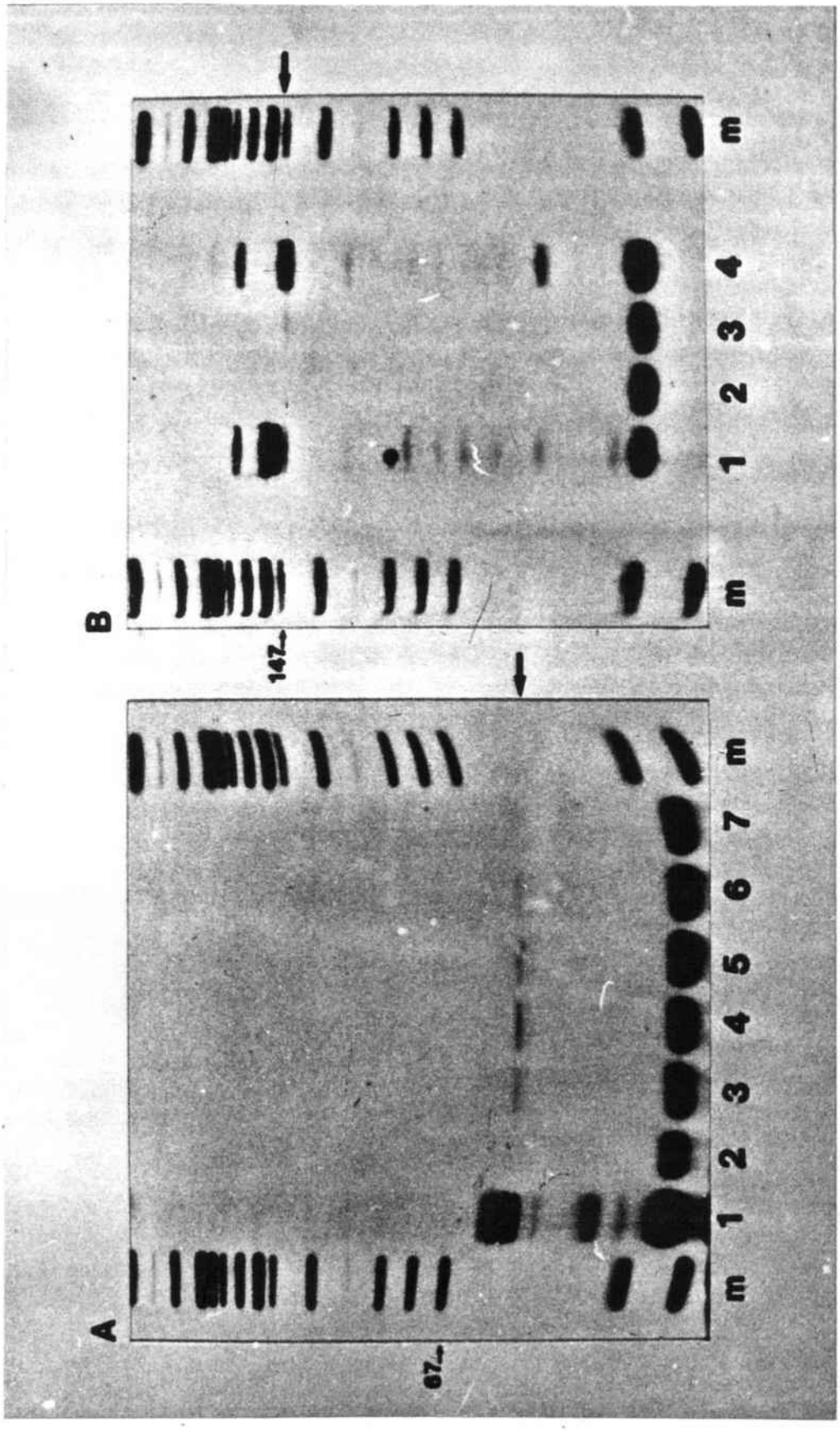

A
B
147
76
m
1
2
3
m
1
2
3
4

Figure 13: Effect of a sea urchin gastrula protein fraction on the transcription of injected Spec 1 gene.

A: oocytes were injected with 0 ng (3), 2 ng (4), 5 ng (5), 10 ng (6) or 20 ng (7) of protein, incubated for 5 h , injected with 2 ng of pSpec 1-1 and incubated again for 5 h prior to extraction of the RNA. Primer extension reactions contained 5 ug of sample RNA and 1 ng of Spec1 specific end-labeled primer. Lane 1: 10 ug of sea urchin gastrula RNA; lane 2: 5 ug of RNA from noninjected oocytes; m: size markers. Arrow: correctly initiated mRNA.

B: as a positive control, another group of oocytes was injected with 0 ng (3) or 10 ng (4) of protein and 5 h later with 4 ng of pSpL1. Times of incubation and conditions for primer extension were as in A, except that the LH2b specific primer was used instead of the Spec primer. Lane 1: 1 ug of sea urchin gastrula RNA; lane 2: 5 ug of RNA from noninjected oocytes; m: size markers. Arrow: correctly initiated mRNA.


A
B
m 1 2 3 4 5 6 7 m
m 1 2 3 4 m
147
97

BIBLIOGRAPHY

Adamson, E.D. and H.R. Woodland (1974). Histone Synthesis in Early Amphibian Development: Histone and DNA Synthesis are not coordinated. J. Mol. Biol. 88: 263-285.

Ashburner, M., C. Chihara, P. Meltzer and G. Richards (1973). Temporal Control of Puffing Activity in Polytene Chromosomes. Cold Spring Harbor Symp. Quant. Biol. 38: 655-662.

Atkinson, T. and M. Smith (1984). Solid-Phase Synthesis of Oligodeoxyribonucleotides by the Phosphite-triester Method. pp 35-81. In "Oligonucleotide Synthesis, a Practical Approach" M.J. Gait, ed. IRL Press, Oxford.

Banerji, J., S. Rusconi and W. Schaffner (1981). Expression of a B-Globin Gene Is Enhanced by Promoter SV40 DNA Sequences. Cell 27: 299-308.

Bendig, M.M. and J.G. Williams (1984). Fidelity of Transcirption of Xenopus laevis Globin Genes Injected into Xenopus laevis Oocytes and Unfertilized Eggs. Mol. Cell. Biol. 4: 2109-2119.

Benoist, C., K. O'Hare, R. Breathnach and P. Chambon (1980). The ovalbumin gene-sequence of putative control regions. N. A. Res. 8: 127-142.

Birchmeier, C., D. Schumperli, G. Sconzo and M. Birnstiel (1984). 3' Editing of mRNAs: Sequence Requirements and

Involvement of a 60-Nucleotide RNA in Maturation of Histone mRNA Precursors. Proc Natl Acad Sci USA. 81: 1057-1061.

Birnboim, H.C. and J. Doly (1979). A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. N. A. Res. 7: 1513-1523.

Bonner, W.M. (1975 a). Protein Migration into Nuclei I. Frog Oocyte Nuclei In Vivo Accumulate Microinjected Histones, Allow Entry to Small Proteins, and Exclude Large Proteins. J Cell Biol 64: 421-430.

Bonner, W.M. (1975 b). Protein Migration into Nuclei II. Frog Oocyte Nuclei Accumulate a class of Microinjected Oocyte Nuclear Proteins and Exclude a Class of Microinjected Oocyte Cytoplasmic Proteins. J Cell Biol 64: 431-437.

Bradford, M.M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72: 248-.

Breathnach, R. and P. Chambon (1980). Organization and Expression of Eukaryotic Split Genes Coding for proteins. Ann Rev Biochem 50: 349-383.

Briggs, M.R., J.T. Kadonaga, S.P. Bell and R. Tjian (1986). Purification and Biochemical Characterization of the Promoter-Specific Transcription Factior Sp1. Science 234: 47-52.

Briggs, R. and T.J. King (1952). Transplantation of Living Nuclei from Blastula Cells into Enucleated Frog Eggs. Proc Natl Acad Sci USA 38: 455-463.

Brown, D.D. (1984). The Role of Stable Complexes that Repress and Activate Eucaryotic Genes. Cell 37: 359- 365.

Brown, D.D. and E. Littna (1964). RNA Synthesis During the Development of Xenopus laevis the South African Clawed Toad. J. Mol. Biol. 8: 669-687.

Caruthers, M.H. (1982). Chemical Synthesis of Oligo-deoxynucleotides Using the Phosphite Triester Intermediates. pp 71-79. In "Chemical and Enzymatic Synthesis of Gene Fragments". Gassen, H.G. and A. Lang, eds. Verlag Chemie, Deerfield Beach, Florida.

Childs, G., R. Maxson and L.H. Kedes (1979). Histone Gene Expression During Sea Urchin Embryogenesis: Isolation and Characterization of Early and Late Messenger RNAs of Strongylocentrotus purpuratus by Gene-Specific Hibridization and Template Activity. Dev. Biol. 73: 153-173.

Colman, A. and J. Morser (1979). Export of Proteins from Oocytes of Xenopus laevis. Cell 17: 517-526.

Colman, A., C.D. Lane, R. Craig, A. Boulton, T. Mohun, J. Morser (1981). The Influence of Topology and Glycosylation on the Fate of Heterologous Secretory Proteins Made in Xenopus Oocytes. Eur. J. Biochem.

113: 339-348.

Davidson, E.H. (1986). Gene Activity in Early Development. 3d ed. Academic Press, Orlando.

Dignam, J.D., P.L. Martin, B.S. Shastry and R.G. Roeder (1983). Eukaryotic Gene Transcription with Purified Components. Meth Enz 101: 582-598.

Dumont, J.N. (1972). Oogenesis in Xenopus laevis (Daudin) I. Stages of Oocyte Development in Laboratory Maintained Animals. J. Morph. 136: 153-180.

Dworkin, M.B., B.K. Kay, J.W.B. Hershey and I.B. Dawid (1981). Mitochondrial RNAs are Abundant in the Poly $(A)^+$ RNA Population of Early Frog Embryos. Dev. Biol. 86: 502-504.

Etkin, L.D. and M.A. DiBerardino (1983). Expression of Nuclei and Purified Genes Microinjected into Oocytes and Eggs, In "Eukaryotic Genes, Their Structure, Activity and Regulation". (N. Maclean, S.P. Gregory and R.A. Flavell, eds.) p.127. Butterworth London.

Etkin, L.D. and M. Roberts (1983). Transmission of Integrated Sea Urchin Histone Genes by Nuclear Transplantation in Xenopus Laevis. Science 221: 67-69

Etkin, L.D., B. Pearman, M. Roberts and S. Bektesh (1984). Replication Integration and Expression of Exogenous DNA Injected into Fertilized Eggs of Xenopus laevis. Differentiation 26: 194-202.

Etkin, L.D. and S. Balcells (1985). Transformed Xenopus

Embryos as a Transient Expression System to Analyze Gene Expression at the Midblastula Transition. Dev. Biol. 108: 173-178.

Etkin, L.D. and B. Pearman (1987). Distribution, Expression and Germ Line Transmission of Exogenous DNA Sequences Following Microinjection into Xenopus laevis Eggs. Development 99

Fried, M. and D.M. Crothers (1981). Equilibria and Kinetics of lac Repressor-Operator interactions by Polyacrylamide Gel Electrophoresis. N. A. Res. 9: 6505- 6525.

Gorman, C. (1982). High Efficiency Transfer into Mammalian Cells p 143. In "Eukaryotic Gene Expression" Kumar, A. ed. Plenum Press, New York.

Gorman, C.M., L.F. Moffat and B. H. Howard (1982). Recombinant Genomes which Express Chloramphenicol Acetyltransferase in Mammalian Cells. Mol.Cell.Biol 2: 1044-1051.

Graves, B.J., P.F. Johnson and S.L. McKnight (1986). Homologous Recognition of a Promoter Domain Common to the MSV LTR and the HSV tk gene. Cell 44: 565 - 576.

Grosschedl, R. and M.L. Birnstiel (1980). Identification of Regulatory Sequences in the Prelude of an H2a Histone Gene by the Study of Specific Deletion Mutants In Vivo. Proc Natl Acad Sci USA 77: 1432-1436.

Gurdon, J.B. and M.P. Wickens (1983). The use of Xenopus Oocytes for the Expression of Cloned Genes. Meth. Enz.

101: 370-386.

Hanahan, D. (1983). Studies on Transformation of Escherichia Coli with Plasmids. J. Mol. Biol. 166: 557-580.

Hardin, S.H., C.D. Carpenter, P.E. Hardin, A.M. Bruskin and W.H. Klein (1985). Structure of the Spec 1 Gene Encoding a Major Calcium-Binding Protein in the Embryonic Ectoderm of the Sea Urchin, Strongylocentrotus Purpuratus. J. Mol. Biol. 186: 243-255.

Harland, R.M., H. Weintraub and S.L. McKnight (1983). Transcription of DNA Injected into Xenopus Oocytes Is Influenced by Template Topology. Nature 302: 38-43.

Harris, S.E., J.M. Rosen, A.R. Means and B.W. O'Malley (1975). Use of a Specific Probe for Ovalbumin mRNA to Quantitate Estrogen Induced Gene Transcripts. Biochem. 14: 2072-2080.

Hawley, D.K. and R.G. Roeder (1985). Separation and Partial Characterization of Three Functional Steps in Transcription Initiation by Human RNA Polymerase II. J Biol Chem 260: 8163-8172.

Heinz, N. and R.G. Roeder (1984). Transcription of Human Histone Genes in Extracts from Synchronized HeLa Cells. Proc Natl Acad Sci 81: 2713-2717.

Hentschel, C., E. Probst and M.L. Birnstiel (1980). Transcriptional Fidelity of Histone Genes Injected into Xenopus Oocyte Nuclei. Nature 288: 100-102.

Ish-Horowicz, D. and J.F. Burke (1981). Rapid and Efficient Cosmid Cloning. N. A. Res. 9: 2989-2998.

Jacob, R. and J. Monod (1961). Genetic Regulatory Mechanisms in the Synthesis of Proteins. J Mol Biol 3: 318-356.

Jones, N., J.D. Richter, D.L. Weeks and D.L. Smith (1983). Regulation of Adenovirus Transcription by E1A in microinjected Xenopus Oocytes. Mol. Cell. Biol. 3: 2131-2142.

Just, L., R. Frankis, W.S. Lowery, R.A. Meyer and G.V. Paddock (1983). Rapid High-Yield Purification of Plasmids by Alkaline Extraction, PEG Precipitation, and Agarose Gel Chromatography. Biotechniques Sept/Oct 83: 136-140.

Kedes, L.H., R.H. Cohn, J.C. Lowry, A.C.Y. Chang and S.N. Cohen (1975). The Organization of Sea Urchin Histone Genes. Cell 6: 359-369.

Kelly, R.B., N.R. Cozzarelli, M.P. Deutscher, I.R. Lehman and A. Kornberg (1970). Enzymatic Synthesis of Deoxyribonucleic Acid XXXII. Replication of Duplex Deoxyribonucleic Acid by Polymerase at a Single Strand Break. J. Bio. Chem. 245: 39-45.

Maniatis, T., E.F. Fritsch and J. Sambrook (1980). Molecular Cloning. A Laboratory Manual. CSH Laboratory, Cols Spring Harbor, New York.

Manley, J.L., A. Fire, A.Cano, P.A. Sharp and M.L. Gefter

(1983). DNA-Dependent Transcription of Adenovirus Genes in a Soluble Wholecell Extract. Proc Natl Acad Sci USA 77: 1180-1184.

Marczynski, G. and J.A. Jaehning (1985). A Transcription Map of a Yeast Centromere Plasmid: Unexpected Transcripts and Altered Gene Expression. N. A. Res. 13: 8487-8506.

Maxam, A.M. and W. Gilbert (1980). Sequencing End-Labeled DNA with Base Specific Chemical Cleavages. Meth. Enz. 65: 499-560.

Maxson, R., T. Mohun, G. Gormenzano, G. Childs and L. Kedes (1983). Distinct Organizations and Patterns of Expression of Early and Late Histone Gene Sets in the Sea Urchin. Nature 301: 120-125.

Maxson, R., M. Ito, S. Balcells, M. Thayer and L. Etkin (1986). Differential Expression of Sea Urchin Early and Late Histone Genes in Xenopus Oocytes in Response to a Trans-Acting Factor Isolated from Late Stage Sea Urchin Embryos. Submitted for Publication.

McMaster, G.K. and G.G. Carmichael (1977). Analysis of Single- and Double-Stranded Nucleic Acids on Polyacrylamide and Agarose Gels by Using Glyoxal and Acridine Orange. Proceedings of the National Academy of Sciences 74: 4835-4838.

McKnight, S.L., E.R. Gravis and R. Kingsbury (1981). Analysis of Transcriptional Regulatory Signals of the

HSV Thymidine Kinase Gene: Identification of an Upstream Control Region. Cell 25: 385-398.

McKnight, S. and R. Kingsbury (1982). Transcriptional Control Signals of a Eukaryotic Protein-Coding Gene. Science 217: 316-324.

McKnight, S. and R. Tjian (1986). Transcriptional Selectivity of Viral Genes in Mammalian Cells. Cell 46: 795-805.

Meinkoth, J. and G. Wahl (1984). Hybridization of Nucleic Acids Immobilized on Solid Supports. Anal. Biochem. 138: 267-284.

Mous, J., H. Stunnenberg, O. Georgiev and M. Birnstiel (1985). Stimulation of Sea Urchin H2b Histone Gene Transcription by a Chromatin-Associated Protein Fraction Depends on Gene Sequences Downstream of the Transcription Start Site. Mol. Cell. Biol. 5: 2764-2769

Niewkoop, P. and J. Faber (1967). Normal Tables of Xenopus laevis (Daudin). 2d ed. Amsterdam: North Holland.

Nishikura, K., S. Goldflam and G.A. Vuocolo (1985). Accurate and Efficient Transcription of Human c-myc Genes Injected into Xenopus laevis Oocytes. Mol. Cell. Biol. 5: 1434-1441.

Norgard, M.V., K. Keem and J.J. Monahan (1978). Factors Affecting the Transformation of Escherichia Coli Strain X1776 by pBR322 Plasmid DNA. Gene 3: 279-292.

Parker, C.S. and T. Topol (1984). A Drosophila RNA

Polymerase II Transcription Factor Specific for the Heat Shock Gene Binds to the Regulatory Site of an hsp 70 Gene. Cell 37: 273-283.

Peacock, S.L., C.M. McIver and J.J. Monahan (1981). Transformation of E.Coli using Homopolymer-Linked Plasmid Chimeras. Biochimica et Biophysica Acta 655: 243-250.

Pelham, H.R.B. (1982). A Regulatory Upstream Promoter Element in the Drosophila hsp 70 Heat Shock Gene. Cell 30: 517-528.

Probst, E., A. Kressmann and M.L. Birnstiel (1979). Expression of Sea Urchin Histone Genes in the Oocyte of Xenopus laevis. J. Mol. Biol. 135: 709-732.

Ptashne, M. (1986). Gene Regulation by Proteins Acting Nearby and at a Distance. Nature 322: 697-701.

Rigby, P.W.J., M. Dieckmann, C. Rhodes and P. Berg (1977). Labeling Deoxyribonucleic Acid to High Specific Activity in vitro by Nick-Translation with DNA Polymerase I. J. Mol. Biol. 113: 237-251.

Roeder, R.G. (1974). Multiple Forms of Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase in Xenopus laevis. J. Biol. Chem. 249: 249-256.

Ryoji, M. and A. Worcel (1984). Chromatin Assembly in Xenopus Oocytes: In Vivo Studies. Cell 37: 21-32.

Shastry, B.S., B.M. Honda and R.G. Roeder (1984). Altered levels of a 5 S Gene-Specific Transcription Factor

(TFIIIA) During Oogenesis and Embryonic Development of Xenopus laevis. J. Biol. Chem. 259: 11373-

Smith, L.D. (1986). Regulation of Translation During Amphibian Oogenesis and Oocyte Maturation. pp 131-150. In "Gametogenesis and the Early Embryo. Gall, J.G. ed. Alan R. Liss, Inc, New York.

Southern, E. (1979). Gel Electrophoresis of Restriction Fragments. Methods in Enzymology 68: 152-176.

Stunnenberg, H. and M. Birnstiel (1982). Bioassay for Components Regulating Eukaryotic Gene Expression: A Chromosomal Factor Involved in the Generation of Histone mRNA 3' Termini. Proc. Natl. Acad. Sci. USA 79: 6201-6204.

Thomas, P. (1983). Hybridization of Denatured RNA Transferred of Dotted to Nitrocellulose Paper. Meth. Enz. 100: 255-266.

Trendelenburg, M.F., H. Zentgraf, W.W. Franke and J.B. Gurdon (1978). Transcription Patterns of Amplified Dysticus Genes Coding for Ribosomal RNA after Injection into Xenopus Oocyte Nuclei. Proc Natl Acad Sci USA 75: 3791-3795.

Voellmy, R. and D. Runnger (1982). Transcription of a Drosophila heat shock gene is heat induced in Xenopus Oocytes. Proc. Nat. Acad. Sci. USA 79: 1776-1780.

Wahli, W., I.B. Dawid, G.U. Ryffel and R. Webber (1981). Vitellogenesis and Vitellogenin Gene Family. Science

212: 298-304.

Walker, M.D., T. Edlund, A.M. Boulet and W.J. Rutter (1983). Cell-Specific Expression Controlled by the 5'-Flanking Region of Insulin and Chymotrypsin Genes. Nature 306: 557-561.

Weinberg, E.S., G.C. Overton, R.H. Shutt and R.H. Reeder (1975). Histone Gene Arrangement in the Sea Urchin Strongylocentrotus purpuratus. Proc. Natl. Acad. Sci. USA. 72: 4815-

White, B.A. and F.C. Bancroft (1982). Cytoplasmic Dot Hybridization. J. Biol. Chem. 257: 8569-8572.

Woodland, H.R. and R.Q.W. Pestell (1972). Determination of the Nucleoside Triphosphate Contents of Eggs and Oocytes of Xenopus laevis. Biochem. J. 127: 597-605.

Wormington, W.M., D.F. Bogenhagen and D.D. Brown (1981). A Quantitative Assay for Xenopus 5 S RNA Gene Transcription in vitro. Cell 24: 809-

Wyllie, A.H., R.A. Laskey, J. Finch and J. B. Gurdon (1978). Selective DNA Conservation and Chromatin Assembly after Injection of SV40 DNA into Xenopus Oocytes. Dev Biol 64: 178-188.