TRANS-STIMULATION OF CHICKEN HISTONE H5 GENE TRANSCRIPTION

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for the degree of Doctor of Philosophy

by

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STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma by any University. To the best of my knowledge, it contains no material that has previously been published by any other person except where due reference is made in the text. I consent to the thesis being made available for photocopying and for loan.

Peter Lance Wigley

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NOTE TO THE READER

Following the journal 'Cell', genus and species names, such as 'Xenopus laevis', and phrases such as 'in vitro' and 'in vivo' are not italicized or underlined in this thesis.

THESIS SUMMARY

Expression analysis was carried out with the gene for the erythroid-specific chicken histone H5. The primary aim of this work was to identify trans-acting factors involved in the transcription of this gene. The Xenopus oocyte was chosen as the assay system for this study, since cell extracts can be co-injected, with DNA, into oocytes, and since it had previously been shown to be useful in the identification of regulatory factors. The results obtained in this study are outlined below.

(1) Transcription of the H5 gene was shown to be accurately initiated in frog oocytes, and parameters of H5 gene expression were defined.

(2) 5' deletions of the H5 gene were used to identify upstream regions involved in determining transcriptional efficiency in injected oocytes. Two such regions were identified - one which inhibited transcription and one which was involved in stimulation of transcription.

(3) In order to identify H5 gene transcription factors, chromatin salt-wash fractions (CSWFs), made from chicken erythroid cells (a transformed cell line, grown in culture) were co-injected, with the H5 gene, into frog oocytes. Other chicken histone genes (H1, H2A and H2B) were also injected in these experiments, to act as internal controls. Primer extension analysis on RNA from injected oocytes indicated that the CSWFs produce an increase in the level of H5 gene transcripts, relative to the transcripts from the control genes.

(4) The H2B gene was cloned next to the H5 gene, in M13. Co-injection of this clone with the CSWFs (and control genes) resulted in an increase in the level of both the H5 and the H2B transcripts. This, together with subsequent experiments, suggested that the CSWFs stimulate H5 gene transcription, and that this effect involves an enhancer-like activity. Furthermore, this effect appears to be mediated by H5 gene sequences.

(5) As an initial investigation into the regions of the H5 gene involved in the trans-stimulation effect, 5' and 3' deletions of the H5 gene were used in CSWF co-injection experiments. The results suggest that a region of the H5 gene between -85 and +313 (relative to the cap site at +1) is sufficient to generate trans-stimulation of H5 gene transcription.

(6) A preliminary study was undertaken into the nature of the stimulatory factor(s) present in the CSWFs. Firstly, treatment of the CSWFs with proteinase K and phenol/chloroform extraction did not reduce the ability of the CSWFs to stimulate H5 gene transcription. Secondly, a nucleic acid fraction from chicken erythroid cell nuclei was also shown to stimulate H5 gene transcription in co-injected oocytes. Finally, treatment of the nucleic acid fraction with RNase reduced the capacity of this fraction to stimulate H5 transcription. This suggests that the stimulatory factor identified in these experiments may be an RNA molecule, or an RNA-protein complex. [This RNA cannot be the H5 mRNA from the chicken erythroid cells since this has an extra 9 bases in the 5' untranslated region, compared with the transcript produced in injected oocytes, due to a polymorphic insertion/deletion.]

(7) A nucleic acid fraction from chicken T cell nuclei was also found to stimulate H5 transcription in co-injected oocytes, suggesting that the stimulatory factor may not be erythroid cell-specific.

Finally, in addition to the work described above, a study was also undertaken to examine some of the functional properties of H5 protein. Co-injection of H5 protein into oocytes, with plasmids containing three chicken histone genes, resulted in the inhibition of transcription from a cryptic RNA polymerase II promoter located within the histone gene cluster.

CHAPTER 1

INTRODUCTION

CHAPTER 1 : INTRODUCTION

According to widely held epigenetic theories, the phenotype of an organism results from a complex interaction between its genotype and the environment (Dawkins, 1982). For multicellular organisms, genotypic effects are believed to be mediated by selective gene expression (Davidson, 1968; Alberts et al., 1983). Under appropriate conditions, this brings about the regulated development of an organism and maintains the differentiated state of its tissues. The work presented in this thesis focuses on one example of selective gene expression, viz., transcriptional control of the gene for the erythroid-specific chicken histone H5. This chapter attempts to put the experimental work into context and deals with three major areas which are directly relevant to this work. These are: the control of eukaryotic gene transcription (particularly the role of trans-acting factors), the Xenopus oocyte expression system, and histone H5.

1-1 Control of Gene Expression

Cell specialization involves the differential expression of sets of genes in distinct cell types (Davidson, 1968; Alberts et al., 1983). This selective gene expression must be regulated during the development of an organism and during the differentiation of particular cell lineages. In addition, for an organism to function its cells must respond to environmental and physiological stimuli by changing their patterns of gene expression. To effect these changes in gene expression, control mechanisms must operate which act on the pathway from gene to mature gene product (RNA or protein).

1-1-1 Levels of Control

Several control points along the pathway from DNA to mature protein have been identified. These are discussed individually below.

(1) Transcription

Most research has focused on control at the transcriptional level, since transcription is the primary event in the pathway of gene expression, and is believed to be a major level of control (Darnell, 1982; Alberts et al., 1983). Control of transcription will be discussed more fully later in this section and in the subsequent three sections (1-2, 1-3 and 1-4).

(2) RNA processing

For RNA polymerase II genes, processing of most primary transcripts involves addition of a cap at the 5' end, cleavage and poly A addition at the 3' end and removal of introns (Darnell, 1982; Nevins, 1983). Many examples of control at the level of (a) 3' end selection and (b) differential splicing have now been found. These include: (a) the choice of different 3' ends/poly A addition sites to specify the membrane-bound or secreted forms of IgM heavy chains (Early et al., 1980) and the two transcripts from the Drosophila glycinamide ribotide transformylase gene (Henikoff et al., 1983), and (b) differential splicing of the rat calcitonin gene (Rosenfeld et al., 1983), the rat troponin T gene (Medford et al., 1984) and the bovine preprotachykinin gene (Nawa et al., 1984). Several of these examples involve regulation of mRNAs in a tissue-specific manner.

(3) mRNA stability

In general, there is known to be a wide variation in the half-lives of different specific mRNAs in the same cells and under different conditions (Darnell, 1982). Two striking examples of regulation of gene expression at the level of mRNA stability are: the increase of casein mRNA half-life by 17-25 fold in the presence of prolactin (Guyette et al., 1979) and enhancement of vitellogenin mRNA stability by oestrogen, in which there is an increase in half-life from 16 hours to approximately 500 hours (Brock

and Shapiro, 1983).

(4) Translation

Several examples also exist for gene regulation by differential translation. Perhaps the best known case involves the storage of maternal mRNAs by developing oocytes of all animal species (Richter and Smith, 1984). These mRNAs are then translated during development of the embryo. More specific examples include preferential translation of heat shock messages during exposure of Drosophila cells to high temperatures (McGarry and Lindquist, 1985) and enhancement of translation of the yeast GCN4 mRNA by amino acid starvation (Hunt, 1985).

(5) Post-translational processing

Regulation can also occur post-translationally in the form of proteolytic processing of precursor molecules. Many hormones and neuropeptides are synthesised as parts of large precursors which often contain several different active peptides. One of the best characterised examples is that of pro-opiomelanocortin, a precursor which includes within it the sequences of ACTH, MSH and beta-endorphin (Herbert and Uhler, 1982). Most interestingly, this precursor is processed to yield different combinations of peptides in the various tissues in which it is synthesised.

As well as the aforementioned levels of control, other, perhaps less common, regulatory mechanisms have been identified in eukaryotic cells. These include rearrangement of DNA sequences (immunoglobulin genes - Tonegawa, 1983), amplification of genes (eg. ribosomal genes - Brown and Blackler, 1972) and DNA transposition (eg. trypanosome VSG genes - Bernards, 1984). There also exists the potential for control at other levels, such as nucleo-cytoplasmic transport of mRNAs.

1-1-2 Transcriptional Control

The preceding discussion illustrates the diversity of regulatory mechanisms which operate to control eukaryotic gene expression. As mentioned above, however, it appears likely that the major form of control operates at the level of transcription. In particular, control of transcription initiation appears to be especially important (Darnell, 1982). This control involves two principal aspects, viz., (1) when and where transcription of a particular gene will be initiated, and (2) the actual mechanism of transcription initiation and modulation of the rate of this process. Of course, these two aspects actually represent different events along the one pathway leading to appropriately regulated gene expression. At this stage most research, including the work presented in this thesis, has been directed towards understanding the more immediate aspects of the control of gene transcription. The next three sections of this introduction focus on the mechanisms of transcription initiation.

This discussion is broadly based on the idea that gene activation in eukaryotes involves two major steps (Alberts et al., 1983). Firstly, the chromatin into which the gene is packaged becomes 'open' to allow access to transcription factors. Secondly, these factors bring about initiation of transcription of the gene. This in turn involves two aspects: the DNA sequences with which the factors interact, and the factors themselves. The following discussion considers these three areas - 'active' chromatin, DNA sequence elements and trans-acting factors. Because the concern of this thesis is the erythroid-specific histone H5, particular emphasis will be placed on examples of tissue-specific gene expression.

1-2 Active Chromatin

Histone proteins package the DNA of eukaryotic organisms into chromatin (Kornberg, 1977; Igo-Kemenes et al., 1982; Weisbrod, 1982). This

packaging involves several levels of structure, from the basic unit, the nucleosome (Richmond et al., 1984), through higher order structures, to the chromosome. At least some of these higher order structures are believed to require the linker histone H1, or its variants, for their formation (Weintraub, 1984; Hannon et al., 1984).

Since it is known that active genes are still associated with histones (Weintraub, 1985), it seems clear that the first step in gene activation must be to generate an open chromatin state, to allow the interaction of transcription factors with the DNA. Moreover, there is now evidence that histone H1, due to its role in the formation of higher order chromatin structures, is a general repressor of gene activity (Weintraub, 1984 and 1985). It is thought that this mechanism ensures the strict suppression of inappropriate gene transcription, whereas other mechanisms exist to turn on specific genes in particular cell types and/or under particular conditions.

There is now a large amount of evidence which indicates that the chromatin of active genes exists in an altered, accessible, conformation (Weisbrod, 1982; Elgin, 1984). Aside from the general distinction between euchromatin and heterochromatin, more specific data have been collected concerning increased nuclease sensitivity of active genes (particularly the occurrence of DNase I and S1 hypersensitive sites), the roles of torsional stress and altered DNA structures, nucleosome phasing, and association of active genes with the nuclear matrix.

1-2-1 DNase I Hypersensitive Sites

Active genes are generally more sensitive to DNase I digestion than inactive genes throughout the entire region of the gene (Mathis et al., 1980). However, more attention has focused on the finding that active genes are usually flanked at their 5' ends by DNase I hypersensitive sites

(DHSs; Elgin, 1984). Many studies suggest that these sites are intimately associated with the potential or actual transcription of the gene under investigation.

Different sets of DHSs have been found upstream from the chicken lysozyme gene, depending on the different functional states of the gene in various tissues (Fritton et al., 1984). Also, as in other systems, one of the DHSs appears and disappears as a consequence of the presence or absence of steroid hormones. In the case of glucocorticoid induction of transcription from the mouse mammary tumour virus promoter, the induced DHS coincides with a region that specifically binds purified glucocorticoid receptor in vitro (Zaret and Yamamoto, 1984). The oestrogen-responsive chicken vitellogenin gene also exhibits different DHSs in different tissues and in the presence or absence of steroid hormones (Burch and Weintraub, 1983). Also, two of the vitellogenin gene DHSs, although induced by oestrogen, still remain after hormone withdrawal, indicating that a stable change in chromatin structure has occurred.

Globin genes have also been the focus of a great deal of research on DHSs. Weintraub et al. (1982), using chicken erythroid cell lines, showed that a switch to produce haemoglobin was accompanied by the acquisition of DHSs by the globin genes under study. Furthermore, one particular cell line had already acquired globin DHSs but these genes had not yet begun transcription. This suggests that changes in chromatin structure, such as DHS formation, may precede transcription and are not necessarily a consequence of it.

The mechanisms which generate DHSs are largely unknown, but several studies suggest the involvement of trans-acting factors, particularly DNA binding proteins. Weisbrod et al. (1980) found that HMGs 14 and 17 can sensitize globin genes to DNase I. More specifically, as discussed above,

the binding of activated steroid hormone receptors is associated with DNase I hypersensitivity of responsive genes (Zaret and Yamamoto, 1984). Recently, Emerson and Felsenfeld (1984) isolated protein factors, present in nuclear extracts from 9 day or adult chicken erythrocytes, which impart hypersensitivity to chicken adult beta-globin genes. DHS formation is observed when these factors are assembled, with histones, on plasmids containing the globin gene, and the hypersensitive region corresponds to sequences sensitive to DNase I in vivo. Moreover, footprinting studies have demonstrated binding of the nuclear factors to regions within the hypersensitive domain (Emerson et al., 1985).

1-2-2 S1 Hypersensitive Sites and Altered DNA Structures

As well as DHSs, 5' regions of some active genes exhibit sites hypersensitive to S1 nuclease (Larsen and Weintraub, 1982). These S1 hypersensitive sites (SHSs) are also present in supercoiled plasmids which carry gene sequences, and are believed to represent regions of altered DNA structure (Nickol and Felsenfeld, 1983; Evans et al., 1984). It is possible that different DNA conformations act as signals for regulatory factors involved in transcription, or simply prevent nucleosome formation and thus increase access of the transcription machinery to a promoter region.

1-2-3 Supercoiling

Several studies suggest that DNA supercoiling may play an important role in eukaryotic gene expression. As mentioned above, in vitro SHS formation is dependent upon the supercoiled state of gene-containing plasmids. Villeponteau et al. (1984) also found that a topoisomerase II-inhibitor, novobiocin, can reverse the DNase I sensitivity of chicken globin genes in vivo. The most convincing evidence concerning the role of supercoiling comes from the extensive studies by Worcel's group, using

Xenopus oocytes. These studies have demonstrated that circular DNA molecules, injected into oocyte nuclei, are assembled into two different forms of chromatin. The transcriptionally active mini-chromosomes display torsionally strained, 'dynamic' DNA supercoils, whereas the inactive molecules do not (Ryoji and Worcel, 1984). Formation of dynamic chromatin requires DNA binding proteins, which appear to compete with histones for initial binding to the DNA (Gargiulo et al., 1984). Moreover, the 5S RNA gene transcription factor, TFIIIA, induces DNA gyration in oocyte extracts (Kmiec and Worcel, 1985).

1-2-4 'Open' Nucleosomes

The studies reported by Worcel's group also suggest that nucleosomes on transcriptionally active DNA differ in structure from those on inactive regions (Ryoji and Worcel, 1985). Specifically, it seems likely that histones on active genes are in a 'half-nucleosome' form, in which the symmetrical halves of the nucleosome are separated to generate a more open conformation. The half-nucleosome is very similar in its properties to the 'lexosome', an open nucleosome structure which is believed to occur on transcribing regions of Physarum ribosomal genes (Prior et al., 1983).

Several factors may be involved in the generation of such open nucleosomes. These include: histone modifications, a deficiency of histones H2A and H2B (Baer and Rhodes, 1983), interactions with HMG proteins, and binding of specific transcription factors (Ryoji and Worcel, 1985).

1-2-5 Nucleosome Phasing and Nuclear Matrix Association

Specific phasing of nucleosomes on DNA and preferential association of active genes with the nuclear matrix are two phenomena which may be important for gene expression, but about which the evidence remains controversial.

There is now a large amount of evidence that, at least in some cases, nucleosomes are non-randomly positioned on DNA sequences (Zachau and Igo-Kemenes, 1981; Linxweiler and Horz, 1985). Furthermore, Strauss and Varshavsky (1984) have reported the identification of a nucleosome-positioning protein from African green monkey cells. However, despite these data, the importance of nucleosome phasing is currently open to question. The major reasons for this are the possibility of artifacts in some phasing experiments (McGhee and Felsenfeld, 1983) and the fact that no biological significance has yet been demonstrated for specific nucleosome positioning (Weintraub, 1985).

The chromatin of eukaryotic cells appears to be organised into loops or domains, which are constrained by a structure known as the nuclear matrix or scaffold (Mirkovitch et al., 1984). Some evidence suggests that active genes are preferentially associated with the nuclear matrix (eg. Ciejek et al., 1983). However, at this stage there is still debate as to the validity of the procedures used in matrix-association experiments (Zakian, 1985). While this debate continues it is difficult to draw conclusions concerning the importance of nuclear matrix interactions.

1-2-6 Summary

It is clear from the above discussion that the chromatin of active genes exists in an altered, more accessible, form. Many factors may be responsible for the generation of active chromatin, including, as discussed, altered DNA structures and DNA binding proteins. Other factors which have not been mentioned, such as DNA methylation (Bird, 1984), may also play a crucial role.

Perhaps the most important aspect of research on chromatin structure is the finding that inactive and active chromatin states can be propagated

to daughter cells in a stable fashion (Weintraub, 1985). This highlights the need to identify more precisely the factors responsible for the generation and maintenance of such states.

1-3 DNA Sequences Involved in Transcription

Identification of DNA sequences involved in the regulation of eukaryotic transcription has been a major focus of research in recent years. Much is now known about many different classes of sequence elements and this section presents a brief summary of the nature and properties of some of these elements.

1-3-1 Prokaryotes

A consideration of the sequences involved in eukaryotic transcription must include reflection on regulatory elements in prokaryotes, since important similarities and differences have been discovered. In general, promoters and regulatory elements for the initiation of bacterial gene transcription are found immediately 5' to the transcription start site. The two most highly conserved elements are the Pribnow box, found at -10, with respect to the transcription initiation site (+1), and the -35 region, which represent interaction sites for RNA polymerase (Siebenlist et al., 1980). Interestingly, the consensus sequence for the Pribnow box, TATAAT (all sequences in this section read 5' to 3' on the antisense strand), is very similar to that for the 'TATA box' of eukaryotic RNA polymerase II genes (see below). Elements responsible for positive and negative regulation of the basic promoter are often found very close to the promoter region (Schaffner, 1985). As will be seen, control of eukaryotic transcription by DNA sequence elements is similar, but more complex, than that in prokaryotes.

1-3-2 Eukaryotes

Three different classes of eukaryotic genes can be defined, relating

to the type of RNA polymerase which carries out transcription. RNA polymerase (RNAP) I transcribes ribosomal genes, RNAP II transcribes protein-coding genes and RNAP III principally transcribes genes for 5S RNA and tRNA molecules. This overview of eukaryotic regulatory sequences will begin with a brief discussion on RNAP I and III genes but will then focus predominantly on elements involved in transcription of RNAP II genes. These latter elements fall into several different categories but, as will become clear, these overlap extensively.

Data concerning which sequences are important for eukaryotic transcription have been principally collected in two ways. Firstly, DNA sequencing of genes has revealed regions which are conserved among many other genes in their sequence and/or position, or are conserved among genes of the same type, or with the same expression properties. Secondly, the functional significance of gene regions or specific sequence motifs has been demonstrated by the use of suitable gene expression systems, often in combination with mutagenesis of the sequences under study. Details of techniques utilised in these experiments will not be discussed here; however a discussion of expression systems is given in section 1-5. Also, discussion of the trans-acting factors responsible for the function of some of the sequence elements mentioned below is presented in section 1-4.

1-3-3 Genes Transcribed by RNA Polymerases I and III

(1) RNAP I genes

Sequences sufficient to promote transcription of Xenopus ribosomal genes in Xenopus oocyte nuclei or in oocyte nuclear extract have been defined to a region from -142 to +6, relative to the transcription initiation site (Sollner-Webb et al., 1983). Similarly, elements of the Drosophila ribosomal gene promoter lie within the region from -43 to +4

(Kohorn and Rae, 1983). As well as these sequences, repeated elements within Xenopus ribosomal gene spacers have been shown to stimulate rDNA transcription, in a manner which suggests similarities with RNAP II gene enhancers (Labhart and Reeder, 1984; see 1-3-4 (4)).

(2) RNAP III genes

The sequence regions principally required to promote initiation of RNAP III gene transcription are located within the transcribed portion of the genes. For 5S RNA genes, the intragenic promoter region lies between +50 and +83 (Korn, 1982). RNAP III initiates transcription at a fixed distance upstream from this region. The internal promoter of tRNA genes consists of two essential regions of about 10 nucleotides, separated by 30 to 40 base pairs (bps). These two regions have been termed box A and box B and RNAP III initiates transcription 11-18 bps upstream from box A (Ciliberto et al., 1983).

1-3-4 Genes Transcribed by RNA Polymerase II

(1) General initiation elements

The initiation site, or cap site, of RNAP II gene transcription is often an A residue, surrounded by pyrimidines (Breathnach and Chambon, 1981). Aside from this, no consensus sequence appears to exist for this region.

Almost all RNAP II genes so far studied have an A-T rich region at approximately -30 with respect to the cap site. This element, termed the TATA box, usually consists of about 6 or 7 A-T bps in succession, with a consensus sequence being T-A-T-A-A/T-A-A/T (Breathnach and Chambon, 1981). However, the precise sequence of this element is not well conserved and some genes completely lack a recognisable TATA box (Baker et al., 1979). Mutation and expression studies have defined the TATA box as an element primarily responsible for selection of the transcription initiation site,

although it may also contribute to the rate of initiation (Grosschedl and Birnstiel, 1980).

(2) Common 5' modulator elements

An element found upstream of many eukaryotic genes is the CAAT box. This motif is usually positioned at approximately -80 and has a consensus sequence of G-G-C/T-C-A-A-T-C-T (Benoist et al., 1980). The functions of sequences similar to this motif differ quite dramatically in different gene systems. Dierks et al. (1983) found that the CAAT box of the rabbit beta-globin gene was important for maximal transcription after transfection into mouse cells. Similarly, a sequence with homology to the CAAT box is a positive promoter element of the herpes simplex virus thymidine kinase (HSV TK) gene (McKnight et al., 1985). However, deletion of a region of the sea urchin H2A gene, which contains two copies of a CAAT box-like sequence, actually increased transcription approximately two-fold (Grosschedl and Birnstiel, 1980). Finally, a mutation in a CAAT box of a human gamma-globin gene was associated with hereditary persistence of fetal haemoglobin in one case studied (Gelinas et al., 1985).

The GC box is a regulatory element found upstream of many viral and cellular genes (Dynan and Tjian, 1985). These genes contain one or more copies of the hexanucleotide sequence GGGCGG or CCGCCC, the best studied examples being the SV40 promoter region (Gidoni et al., 1984) and the HSV TK gene (McKnight et al., 1984). In the cases studied, the GC box is important for efficient transcription and can exert its effect in an orientation-independent manner (Dynan and Tjian, 1985).

(3) Intragenic elements

As is the case with RNAP III genes, some RNAP II genes contain sequences within the transcribed portion of the gene which are important

for efficient transcription. The best known example is the enhancer located in the major intron of immunoglobulin genes (e.g. Gillies et al., 1983). Other intragenic sequences which appear to affect transcription include: a glucocorticoid regulatory element in the first intron of the human growth hormone gene (Slater et al., 1985) and sequences within human alpha- and beta-globin genes which are sufficient for regulated expression of these genes following introduction into mouse erythroleukemia cells (Charnay et al., 1984).

(4) Enhancers

Enhancers are cis-acting elements which can stimulate transcription over long distances and in an orientation-independent manner (Schaffner, 1985). They have been found in many viral and cellular genes and can exert their effect on homologous or heterologous promoters. Also they function when located either 5' or 3' to initiation sites. The 'prototype' enhancer is the 72 bp tandem repeat of SV40 (Banerji et al., 1981), which is the best characterised of these elements. Active research is also focused on many other enhancers, such as the immunoglobulin gene enhancer (Gillies et al., 1983) and the enhancer of human cytomegalovirus, which appears to be the strongest element of this type so far analysed (Boshart et al., 1985). No one sequence motif is found in all enhancers, but rather it seems that many different sequences may be able to function as transcriptional enhancers. The mechanism of enhancer function is unknown, but it appears likely that enhancer-binding proteins may play a role in the enhancement effect. These proteins may also be responsible for the host and cell-type preferences displayed by many enhancers (Schaffner, 1985).

(5) Gene-specific elements and Inducible genes

Many genes of the same type have been found to contain homologous sequences at similar positions upstream from the cap site (Davidson et al.,

1983). These sequences are all approximately 10 to 20 nucleotides in length and are thought to be involved in the regulation of gene expression. For some of these gene-specific sequences, it is difficult even to postulate functions; examples are the specific elements of histone H1 genes (Coles and Wells, 1985) and histone H2B genes (Harvey et al., 1982). Other elements are found upstream of genes which are induced by the same environmental or physiological stimuli, such as heat or steroid hormones (Davidson et al., 1983). In these examples the functional significance of the specific elements seems obvious and in several cases this significance has been demonstrated experimentally.

Examples of inducible transcription include: glucocorticoid induction of the mouse mammary tumour virus promoter (Chandler et al., 1983) and the human metallothionein-IIA gene (Karin et al., 1984), heavy metal induction of the same metallothionein gene (Karin et al., 1984), activation of heat shock gene transcription (Pelham, 1982), interferon-induced transcription of HLA and metallothionein genes (Friedman and Stark, 1985), light regulation of plant gene expression (Timko et al., 1985) and viral or poly (I)-poly (C) activation of human interferon gene transcription (Goodbourn et al., 1985). Most of these cases involve sequence elements which are conserved among similarly regulated genes and many of these regulatory sequences have the properties of transcriptional enhancers. The presence of these gene-specific elements suggests that a common regulatory factor can be involved in the induction of a set of linked, or unlinked, genes (Davidson et al., 1983).

(6) Sequences involved in tissue-specific gene expression

As discussed in section 1-1, many of the specific characteristics of distinct cell types result from tissue-specific gene expression.

Furthermore, transcription initiation is a major level of control of this differential expression (Darnell, 1982; Alberts et al., 1983). Sequences responsible for the cell type-specific transcription of many genes have now been characterised.

The first genetic element to be implicated in tissue-specific gene expression was the immunoglobulin heavy chain enhancer (eg. Gillies et al., 1983). This enhancer lies in the intron between the J and C segments of the immunoglobulin gene, 3' to the cap site. An enhancer conferring lymphoid cell-specific expression has also been identified in light chain genes (eg. Picard and Schaffner, 1984). Recent data suggest that the enhancer is only one of three elements involved in cell type-specific expression of immunoglobulin heavy chain genes; other intragenic sequences and an upstream promoter region can also direct selective expression (Grosschedl and Baltimore, 1985).

Sequences responsible for the regulated expression of globin genes in mouse erythroleukemia cells have been identified (Charnay et al., 1984; Wright et al., 1984). Interestingly these sequences were found to be located both 5' and 3' to the translation initiation site.

A more precise characterisation has been undertaken of the elements sufficient for cell type-specific transcription of insulin and chymotrypsin genes (Walker et al., 1983). For the rat insulin gene these sequences lie between -302 and +51, for the human insulin gene the region is from -258 to +241 and for the rat chymotrypsin gene the sequences are located between -274 and -3. These regulatory sequences also exhibit the properties of enhancer elements.

Similar experiments have localized sequences mediating the specific expression of the chicken alpha-crystallin gene in mouse lens cells. The regulatory region lies between -242 and -189 and also acts in a true

enhancer-like fashion (Okazaki et al., 1985).

The studies described above have utilised the introduction of genes into specific cell types in culture in order to characterise sequences mediating tissue-specific expression. Another major approach has been to introduce gene sequences into the germ line of Drosophila or mice and test for appropriate cell type-specific regulation. In the case of Drosophila, the genes xanthine dehydrogenase, alcohol dehydrogenase and dopa decarboxylase were introduced into the germ line via P-element transformation. The tissue-specificity of the expression of all three genes was found to be the same as that seen in wild-type flies (Flavell, 1983). After introduction into mice, several genes have also been shown to be expressed in a cell type-specific manner. These include the rat elastase I gene, the human beta-globin gene and the mouse kappa light chain immunoglobulin gene (Palmiter and Brinster, 1985). In the case of the elastase gene, the important regulatory sequences were localized to a 213 bp region contiguous with the promoter (Ornitz et al., 1985).

In summary, then, a number of discrete sequence regions have been characterised which mediate cell type-specific expression. Furthermore, many of these regions have the properties of transcriptional enhancers.

(7) Negative regulatory regions

Most of the regulatory elements which have been identified to date mediate positive control of gene transcription. However, some negative regulatory regions have also been characterised.

Several cases of negative regulation have been found with yeast genes (Guarente, 1984; Brent, 1985). In one such case, sequences mediating glucose repression of an alcohol dehydrogenase gene have been localized to an upstream region (Guarente, 1984). Another example involves repression

of the silent genes present at the mating type locus of yeast. One of the cis-acting regions required for this repression, HMRE, has been shown to have properties opposite to those of an enhancer and was thus termed a 'silencer' (Brand et al., 1985).

Negative regulatory elements have also been identified in higher eukaryotes. An upstream region of the mouse beta-major globin gene was found to inhibit transcription of globin promoters in transient expression assays (Gilmour et al., 1984). Interestingly, this element contains a region potentially capable of adopting a Z-DNA conformation. A negative element with a specific function has been identified in the 5' flanking region of the hamster HMG CoA reductase gene. This region contains promoter sequences and sequences which are responsible for cholesterol-mediated inhibition of transcription (Osborne et al., 1985).

1-3-5 Summary

Many sequence elements involved in the control of transcription in eukaryotes have now been identified and, in some cases, these have been characterised in detail. These elements often lie close upstream of a gene and often exhibit the properties which have been defined for transcriptional enhancers. In general, it appears that eukaryotic promoter and regulatory elements have a modular arrangement, being composed of short sequence motifs, each with a specific function. These activities include the mediation of inducibility by various environmental and physiological signals, developmental regulation and cell type-specificity (Schaffner, 1985).

1-4 Trans-acting Factors

It seemed logical, prior to experimental verification, that trans-acting factors, particularly specific DNA binding proteins, would be involved in the regulation of eukaryotic gene transcription. Firstly (by

analogy), control of transcription in prokaryotes is mediated by proteins which bind to specific DNA sequences (discussed below). Secondly, the cytoplasm of one eukaryotic cell type can influence the expression of genes in the nucleus of another cell type. For example, mouse muscle cells fused with human non-muscle cells can produce stable heterocaryons in which the nuclei are distinct, and this fusion induces expression of muscle-specific genes in the non-muscle cell nuclei (Blau et al., 1983). Thirdly, studies in which genes introduced into cells are appropriately induced and/or regulated (as described in section 1-3) suggest the presence of factors in these cells which interact with gene sequences. Furthermore, the identification of conserved DNA sequence elements which are responsible for transcriptional regulation suggests the presence of sequence-specific DNA binding proteins.

In recent years a number of eukaryotic proteins have been identified which bind to specific DNA sequences and/or play a role in the control of transcription. Most cases involve proteins which are contained within crude cell extracts but some essentially pure regulatory proteins have now been isolated.

1-4-1 Techniques for Identifying Regulatory Factors

Many different techniques have been used to identify specific DNA binding proteins and factors which are involved in the mechanism, or regulation, of transcription. These include: nitrocellulose filter-binding assays (Jack et al., 1981), affinity chromatography (Weideli and Gehring, 1980), in vitro transcription (Dynan and Tjian, 1983a), differential band migration on polyacrylamide gels (Piette et al., 1985), protein blotting (Miskimins et al., 1985) and footprinting assays utilising DNase I (Galas and Schmitz, 1978), chemical modification (Gidoni et al., 1984), genomic

sequencing (Nick and Gilbert, 1985), exonuclease III (Wu, 1984) and light ('photo-footprinting'; Becker and Wang, 1984). Some of these methods can only be used in vitro, whereas several of the footprinting techniques can detect specific protein-DNA interactions in vivo.

1-4-2 Prokaryotes

The most detailed studies of proteins involved in transcriptional regulation have been carried out with prokaryotes. The structures of the cI and cro repressors of bacteriophage lambda and the catabolite gene activator protein (CAP) of E. coli have all been elucidated using X-ray crystallography (North, 1984). Furthermore, Anderson et al. (1985) have determined the X-ray crystal structure, at 7Å resolution of the phage 434 repressor bound to its operator DNA. Studies with all of these proteins have demonstrated that each contains a pair of alpha-helices, one of which lies in the major groove of the DNA when bound (North, 1984; Anderson et al., 1985).

The cI repressor of lambda is also a positive regulator of transcription. Evidence suggests that this function is mediated by contact between operator-bound repressor and RNA polymerase (Hochschild et al., 1983).

Another type of prokaryotic protein involved in transcriptional control is the sigma factor. This protein binds to the core RNA polymerase and enables it to accurately initiate transcription. In B. subtilis and Streptomyces coelicolor different sigma factors are utilised to direct initiation of transcription at different classes of promoters (Travers, 1985).

1-4-3 Eukaryotic RNAP I and RNAP III Genes

(1) RNAP I genes

Identification of RNAP I gene transcription factors is still at a

preliminary stage, but several groups have begun to approach this problem (Sommerville, 1984). Perhaps the most interesting finding to date is that some transcription factors for rDNA genes are species-specific - these genes are only transcribed by in vitro systems if certain specific components are from the same species as the gene (Sommerville, 1984).

(2) RNAP III genes

Some of the most detailed studies of eukaryotic transcription factors have been carried out with 5S RNA genes. RNAP III recognises the internal control region of 5S RNA genes in the context of a complex involving at least three transcription factors: TFIIIA, TFIIIB and TFIIIC (Enver, 1985). TFIIIA is specific for 5S genes and binds first to the control region, followed by the sequential binding of factors C and B (Bieker et al., 1985). TFIIIA has been the subject of extensive experimentation, culminating recently in the isolation and sequencing of a TFIIIA cDNA clone, from a Xenopus laevis oocyte library (Ginsberg et al., 1984).

5S DNA transcription complexes formed in vitro are stable, such that many rounds of RNA synthesis can occur without dissociation of the complex (Brown, 1984). In Xenopus somatic cells, only the somatic-type 5S genes are active, whereas the oocyte-type 5S genes are silent. This differential regulation of transcription is dependent upon levels of TFIIIA (Brown, 1984; Brown and Schlissel, 1985). Moreover, in somatic cells, somatic-type 5S genes are packaged in stable transcription complexes, while the oocyte-type genes do not have transcription factors bound to them and are prevented from binding these factors by a structure dependent on histone H1 (Schlissel and Brown, 1984).

From structural analyses of TFIIIA, it appears that this protein is composed of nine flexibly linked, small, looplike domains; these are the

proposed DNA binding regions (Enver, 1985). Furthermore, these protein domains, which interact with metal ions such as zinc, have features in common with regions of many other regulatory and nucleic acid-binding proteins (Berg, 1986). Less is known about the mechanism by which TFIIIA promotes transcription initiation, but, as noted in 1-2-3, it specifically induces DNA gyration of 5S RNA genes in Xenopus oocyte extracts (Kmiec and Worcel, 1985). This activity may be related to the mechanism of action of TFIIIA.

1-4-4 Eukaryotic RNAP II Genes

(1) Yeast

Examples of both positive and negative regulation by trans-acting factors have been characterised in yeast.

Transcription of the GAL1, GAL 7 and GAL10 genes of yeast is induced more than 5000-fold by galactose. This induction depends upon an upstream activating sequence (UAS) and upon the product of the GAL4 gene, which is a positive regulator of transcription (Giniger et al., 1985). Footprinting experiments have demonstrated that this protein binds to four sites in the UAS to activate transcription (Giniger et al., 1985). Furthermore, the action of the GAL4 protein is inhibited by the product of the GAL80 gene and galactose induction is mediated by suppression of the GAL80 effect (Giniger et al., 1985). Finally, the results of recent work suggest that the activity of the GAL4 protein is mediated by protein-protein interactions with other DNA-bound proteins (Keegan et al., 1986).

Repression of transcription by specific proteins is involved in the regulation of yeast mating type. a-specific genes are repressed in alpha mating type cells by a process dependent on the alpha 2 protein. In diploid cells, repression of haploid-specific genes involved both the alpha 2 and a1 protein (Brent, 1985). Alpha 2 protein has been shown to bind to

a site upstream of an a-specific gene (Johnson and Herskowitz, 1985).

(2) Viral transcription factors

Many viruses encode proteins which regulate transcription from viral promoters. Two of the best characterised of these proteins are SV40 T antigen and the E1A protein of adenovirus.

T antigen is encoded by an early gene of SV40 and binds principally to two regions of the viral genome, near the origin of replication (Tooze, 1980). Like the cI repressor of lambda, T antigen is both a positive and negative regulator of transcription. Specific binding of T antigen results in repression of early gene transcription (thereby effecting autoregulation) and binding to the same regions may also be responsible for the ability of T antigen to stimulate late transcription (Ryder et al., 1985).

The 289 amino acid protein encoded by the E1A gene region of adenovirus stimulates transcription from adenovirus promoters (Kingston et al., 1985). Furthermore, E1A has been shown to stimulate transcription from several other, non-adenovirus, promoters. These include the promoters of the human beta-globin gene, the rat preproinsulin gene and the SV40 early region (Kingston et al., 1985). The mechanism by which E1A exerts its positive effect is not yet clearly understood. Deletion analysis has been unable to identify a specific DNA sequence required for E1A stimulation (Kingston et al., 1985). Consistent with this is evidence that E1A proteins do not directly bind to DNA (Ferguson et al., 1985). However, it has recently been shown that E1A increases the efficiency of interaction between a cellular transcription factor and the adenovirus early E2 promoter (Kovesdi et al., 1986). This suggests a possible mechanism for EIA activity. It is also interesting to note that E1A proteins can repress the

stimulation of transcription induced by various enhancer elements (Borrelli et al., 1984).

Recent work has also identified a cellular factor able to stimulate transcription from the major late (ML) promoter of adenovirus (Sawadogo and Roeder, 1985). This factor, which is present in HeLa cell nuclear extracts, acts specifically on the ML promoter and its activity appears to be mediated via binding to a defined upstream region. Moreover, evidence suggests that the stimulatory factor interacts with a TATA box binding factor. An unexpected finding from this work is that the stimulatory factor is extremely heat stable - approximately 80% activity is retained after a 10 minute incubation at 100 ^{o}C.

It should be noted that two other groups have also reported the identification of transcription factors which interact with the adenovirus ML promoter (Carthew et al., 1985; Miyamoto et al., 1985).

(3) General transcription factors

Several factors have been identified which are involved in the transcription of a number of genes and/or bind to common promoter elements in eukaryotes.

Davison et al. (1983) utilised chromatographic fractionation of a HeLa cell in vitro transcription system in order to identify RNAP II transcription factors. One fraction was found to contain a factor, necessary for correct transcription,which bound to TATA box sequences in vitro (see 1-3-4 (1)). Furthermore, this binding resulted in the formation of stable preinitiation complexes, in the absence of RNAP II.

As discussed in 1-3-4 (2), a sequence element related to the CAAT box is a feature of many eukaryotic RNAP II genes. Jones et al. (1985) have identified the presence of a factor in a HeLa cell in vitro transcription system which binds to the CAAT box region of the HSV TK gene. This

interaction is required for optimal transcription of the gene.

One of the best characterised RNAP II gene transcription factors is a protein designated Sp1, which binds to GC box elements (see 1-3-4 (2)). Sp1 was first identified in a fraction from a whole cell HeLa extract, required for transcription of SV40 early and late promoters (Dynan and Tjian, 1983a). This requirement was selective for these promoters over others, such as the human beta-globin, and the adenovirus 2 major late, promoter. This selectivity of Sp1 was subsequently found to be due to its binding in the 21 bp repeat region of SV40 (Dynan and Tjian, 1983b). Further analysis of Sp1 binding was carried out using dimethyl sulphate methylation protection experiments (Gidoni et al., 1984). This showed that the primary binding site of Sp1 is the GC box, GGGCGG, and contacts between Sp1 and this sequence all fall on one strand of the DNA in the major groove of the helix.

Sp1 binding sites are repeated several times in the SV40 promoter region and in a related monkey promoter, which also binds Sp1 (Gidoni et al., 1984). Other promoters have also been found to interact with Sp1, including the promoter of the HSV TK gene (Dynan and Tjian, 1985; Jones et al., 1985). In the case of the TK gene, it has been found that optimal transcription appears to require the coordinate interaction of Sp1 and the CAAT box binding factor with their appropriate binding sites (Jones et al., 1985). Furthermore, it has been suggested that the CAAT binding protein bridges the two Sp1 binding sites, thereby facilitating an indirect interaction between them (McKnight et al., 1985). Finally, it is interesting to note that despite the asymmetry of Sp1 binding to the SV40 promoter region, this region stimulates transcription in a bidirectional manner (Dynan and Tjian, 1985).

Histone genes have also been the subject of research on trans-acting factors involved in transcriptional regulation. Heintz and Roeder (1984) demonstrated that nuclear extracts isolated from synchronised HeLa cells in S phase gave much greater transcription of a human histone H4 gene than extracts from non-S phase cells. This suggests the presence of an S phase-specific trans-acting factor, which may be at least partly responsible for S phase regulation of H4 genes in vivo. Subsequent experiments have confirmed that regulated expression of histone H4 genes in vivo requires a specific, trans-acting, transcription factor (Capasso and Heintz, 1985).

Utilising Xenopus oocyte microinjection, Mous et al. (1985) have characterised a chromatin protein fraction from sea urchin embryos which specifically stimulates transcription of sea urchin histone H2B genes (also see section 1-5-2 (4)). Two regions of the H2B gene appear to be able to mediate this effect, both of which are located downstream from the transcription initiation site.

(4) Enhancer-binding factors

A number of enhancers (1-3-4 (4)) have now been shown to bind specific factors and such interactions are likely to be important for the ability of these elements to potentiate transcription.

Scholer and Gruss (1984) have used competition assays, involving transfection into cells in culture, to identify the presence of cellular factors able to interact with enhancer elements. Different enhancers were found to compete for the same class of factors. However, different competition strengths were observed in the assay and these reflected host cell preferences previously demonstrated for the enhancers. This result suggests that sequence differences in the enhancers may be responsible for differential binding affinities of particular factors and, therefore, differential activity in various cell types. Competition assays were also

carried out by Mercola et al. (1985) to demonstrate the binding of factors to an immunoglobulin heavy chain enhancer. In lymphoid cells, the heavy chain enhancer binds a factor essential for enhancer activity and this factor also binds to the SV40 enhancer. However, a factor present in fibroblasts is utilised by the SV40 enhancer but not by the heavy chain element. These data suggest an explanation for the observed lymphoid cell-specificity of heavy chain enhancer function.

One of the enhancers utilised in the previous experiments was the SV40 72 bp repeat region. Subsequent work by Sassone-Corsi et al. (1985) showed that stable binding of a trans-acting factor is involved in the stimulation of in vitro transcription by this enhancer. This factor can also interact with other enhancer elements.

Experiments with the Xenopus U2 gene have shown that one of its promoter elements stimulates transcription in an enhancer-like manner. This element increases promoter activity by facilitating the formation of stable transcription complexes (Mattaj et al., 1985).

(5) Factors for inducible genes

As described in 1-3-4 (5), the transcription of many genes can be induced by various physiological or environmental signals. The involvement of specific trans-acting factors in this induction has been demonstrated in several cases.

Metallothionein (MT) gene transcription can be induced by various heavy metals, such as zinc, copper and cadmium, and elements upstream of MT genes, which mediate this response, have been identified (Karin et al., 1984). Competition experiments have subsequently been used to detect cellular factors involved in the cadmium induction of the mouse MT-1 gene (Seguin et al., 1984).

Detailed studies have been carried out on trans-acting factors involved in the induction of heat shock genes. The Drosophila hsp 70 gene contains four sites within three domains upstream of the TATA box, to which a heat shock gene-specific transcription factor binds (Topol et al., 1985). Maximal in vitro transcriptional activity of the hsp 70 promoter occurs when all binding sites are present (Topol et al., 1985). Using an exonuclease III footprinting assay, Wu (1984) has analysed regions of Drosophila heat shock genes which are resistant to digestion in vivo. Each of the genes analysed had two resistant upstream domains, one which included the TATA box region and another 5' to this. These results imply the presence of specific factors which bind to, and protect, the sites identified. Most strikingly, the upstream site was found to be resistant to digestion only during heat induction of gene activity, consistent with the binding of a heat shock activator protein.

One of the best characterised of all RNAP II gene transcription factors is the glucocorticoid receptor. The transcription of many genes has now been shown to be inducible by glucocorticoids (and other steroid hormones), and specific DNA sequence elements, which are essential for this effect, have been identified (Karin et al., 1984). These elements function as transcriptional enhancers (Parker, 1983). Using binding and footprinting assays, several groups have demonstrated binding of glucocorticoid receptor to the appropriate regulatory elements of a number of genes. The systems investigated include: the mouse mammary tumour virus promoter (Payvar et al., 1983), the chicken lysozyme gene (von der Ahe et al., 1985), the human metallothionein-IIA gene (Karin et al., 1984) and the human growth hormone gene (Slater et al., 1985). These findings suggest that the binding of activated glucocorticoid receptors to specific DNA sequence elements stimulates transcription of responsive genes. Clearly,

these systems have many features which make them particularly useful models for the analysis of eukaryotic gene regulation. Further support for this claim has been provided by the recent isolation of cDNA clones for glucocorticoid receptors (Miesfeld et al., 1984; Hollenberg et al., 1985).

(6) Factors involved in tissue-specific gene expression

Only a small number of factors involved in the tissue-specific regulation of gene expression have been identified.

Two classes of these factors have already been discussed. Firstly, since genes responsive to steroid hormones are expressed in a tissue-specific manner, steroid hormone receptors can be considered regulators of cell type-specific transcription (see 1-4-4(5)). Secondly, it appears likely that factors which bind to immunoglobulin gene enhancer elements (1-4-4(4)) are involved in the B cell-specific expression of these genes.

As described in section 1-2-1, Felsenfeld and his co-workers have identified factors in erythroid cell nuclear extracts which can generate DNase I hypersensitivity on chicken beta-globin gene chromatin. These factors may be important for the specificity of beta-globin gene transcription. More recently Bazett-Jones et al. (1985) have demonstrated that nuclear extracts from human erythroleukemia-like cells stimulate globin gene transcription in vitro, following addition of the extracts to a HeLa cell-free transcription system. This stimulation is specific for globin genes and for extracts from erythroid cells.

(7) The homeo box

The homeo box is a protein-coding sequence of 180 bps which is highly conserved among a number of Drosophila homeotic genes, and related sequence domains have been identified in a variety of other organisms (Gehring, 1985; Manley and Levine, 1985). Although the significance of the homeo box

is yet to be determined, some evidence suggests that the protein domain encoded by this sequence may function as a sequence-specific DNA binding activity.

Firstly, computer searching through a protein sequence bank has revealed an apparently significant homology between the homeo box protein domain and amino acid regions encoded by the a1 and alpha 2 mating type genes of yeast (Shepherd et al., 1984). This is particularly interesting since the mating type proteins are involved in the determination of specific cell types via transcriptional regulation. Furthermore, as noted in section 1-4-4(1), the yeast alpha 2 protein has been shown to bind to an upstream region of one of its target genes.

Secondly, the homeo box-encoded domain and the related yeast protein regions both have limited homology with several prokaryotic regulatory proteins (Laughon and Scott, 1984). Most importantly, this homology occurs in related regions of the prokaryotic proteins which are strongly implicated as DNA binding domains (these regions include a pair of alpha-helices - see 1-4-2).

Thirdly, Desplan et al. (1985) have demonstrated that a region of the Drosophila engrailed gene which includes the homeo box encodes a protein domain possessing sequence-specific DNA binding activity.

One conclusion which can be drawn from the findings discussed above is that homeo box-containing genes may encode proteins which regulate gene expression during development, via specific DNA-protein interactions. This hypothesis awaits experimental testing.

1-4-5 Summary

Data collected from many gene systems suggest that trans-acting factors, particularly sequence-specific DNA binding proteins, play a fundamental role in the regulation of gene transcription. A number of

different classes of these factors can be distinguished, depending upon the type of sequence element with which they interact and the particular mode of regulation which they mediate.

Aside from the characterisation of other, as yet unidentified, trans-acting transcription factors, two of the most important future aims in this field are to clone the genes for regulatory proteins and to understand the mechanisms by which trans-acting factors function. As mentioned in the main body of this section (1-4), the cloning of regulatory protein genes has already begun. With regard to the second aim, recent evidence suggests that, as is the case with the cI repressor of lambda (see section 1-4-2), protein-protein interactions may be one way in which eukaryotic transcription factors exert their effects (Takahashi et al., 1986; Keegan et al., 1986).

1-5 The Xenopus Oocyte System

The experimental work described in this thesis involves extensive use of the Xenopus laevis oocyte as an assay system. This section presents a dicussion of the Xenopus ooctye focusing on its use in the analysis of transcription and in studies on the interaction of trans-acting factors with co-injected DNA templates.

1-5-1 Gene Expression Systems

A number of systems have been utilised for the analysis of eukaryotic gene expression. These systems will be considered briefly as a background for discussion of the frog oocyte.

(1) In vitro systems

Most of the major steps in the pathway of eukaryotic gene expression can now be studied in various cell-free/in vitro systems. The major advantage of such systems is that active components can be fractionated and

subsequently characterised in detail. Sequence elements involved in the regulation of transcription have been identified using in vitro systems and identification of trans-acting factors which interact with these sequences is an active area of current research (eg. Dynan and Tjian, 1983a; Sawadogo and Roeder, 1985).

(2) Yeast

Powerful techniques have been developed for the introduction of genes into intact yeast cells, including gene replacement by homologous recombination and the construction of artificial chromosomes (Struhl, 1983). These techniques are being utilised in sophisticated studies of yeast gene expression. However, due to differences between the expression of yeast genes and those of higher eukaryotes, yeast is not a generally useful system for the study of eukaryotic gene expression.

(3) Cells in culture

The introduction of genes into cultured cells has been a widely used technique in studies of gene regulation. Several methods are available for gene introduction, including: transfection using various chemical means to facilitate DNA uptake (Spandidos and Wilkie, 1984), microinjection (Capecchi, 1980) and electroporation (Neumann et al., 1982). Following the introduction of genes into cells, transient assays can be carried out on extra-chromosomal gene copies or stable cell lines can be generated after the integration of genes into host chromosomes or with the maintenance of stable episomes (Spandidos and Wilkie, 1984). In these experiments, use is often made of vectors derived from the genomes of viruses such as SV40, BPV and retroviruses (Spandidos and Wilkie, 1984). An important advantage of the use of cultured cells for gene expression studies is that genes can be introduced into their homologous cell types.

(4) Transgenic animals

Techniques have been developed to introduce DNA into the germline of several organisms; the resultant animals being referred to as transgenic. Most work has been carried out with mice (Palmiter and Brinster, 1985) and Drosophila (Flavell, 1983) and microinjection is generally used to produce the transgenic organisms. An obvious advantage of producing transgenic animals is that patterns of gene expression with respect to tissue-specificity can be analysed. Examples of this type of work were discussed in section 1-3-4(6).

1-5-2 Xenopus Oocytes

The Xenopus oocyte system has been the subject of several extensive reviews (Gurdon and Melton, 1981; Wickens and Laskey, 1981; Gurdon and Wickens, 1983; Colman, 1984). The following is a brief outline of this system, with emphasis on factors of particular relevance to this thesis. Most information will be taken from the aforementioned reviews; other references will be provided where appropriate.

(1) General features

Oogenesis in Xenopus laevis has been divided into six stages (Dumont, 1972). Stage VI oocytes are used preferentially for injection. These are very large cells (greater than 1mm in diameter) with two distinct hemispheres, separated by an essentially unpigmented equatorial band. The nucleus (germinal vesicle), which is not visible, occupies a constant position under the apex of the dark hemisphere. Injection aiming at this point allows the introduction of molecules into the oocyte nucleus. The ooctye is in meiotic prophase, is active in RNA and protein synthesis, and inactive in DNA synthesis. It contains large stores of molecules such as the three RNA polymerases and histone proteins.

For the analysis of gene expression, approximately 10^8 to 10^9 circular

DNA molecules are usually injected into the nucleus of each of a batch of 20 to 30 oocytes. These molecules do not integrate, but are assembled into chromatin and exist as discrete 'mini-chromosomes'.

(2) Advantages and Limitations

Many aspects of the frog ooctye make it extremely useful as a gene expression system. Firstly, enough RNA or protein is obtained for analysis from the injection of only a small amount of DNA into a small number of oocytes. In fact the RNA made by a single oocyte is usually sufficient for the analysis of transcription, and this RNA synthesis takes place within 24 hours. Secondly, the injected DNA is assembled into an apparently normal chromatin structure and each DNA molecule exists in a similar genetic environment, unaffected by the presence of adjacent sequences, as would be the case if the DNA integrated into host chromosomes. Thirdly, most of the steps of eukaryotic gene expression are carried out by injected oocytes. Fourthly, a range of different molecules, such as DNA, RNA, proteins and any non-toxic chemicals, can be introduced into oocytes, either singly or in various combinations (see part (4) of this section).

The Xenopus oocyte system also has certain limitations, which may pose problems for particular experiments. Except for studies of Xenopus genes, the oocyte obviously does not provide an homologous system for gene expression. Thus, certain species- or cell type-specific phenomena cannot be investigated using this system. However, the oocyte's lack of certain regulatory factors can also be an advantage, since these factors can then be identified by their co-introduction, with DNA molecules, into the same oocytes (see part (4)). A second possible drawback of the oocyte system is that although injected genes are packaged into chromatin, they are not

contained within a large chromosomal environment. This latter condition may be important for regulation at the level of higher-order structures. Finally, a degree of variability between the oocytes of different frogs has been observed for some effects (eg. Korn and Gurdon, 1981; Jones et al., 1983).

(3) Analysis of transcription

The transcription of many genes has been analysed using the frog oocyte system. These include genes transcribed by RNA polymerases I, II and III. With regard to RNAP II genes, it has been generally established that genes transcribed in many tissues, such as viral genes and histones, are transcribed efficiently in oocyte nuclei. In contrast, some genes normally expressed in a cell type-specific manner are not transcribed well in this system. However, the situation is now known to be more complex. Examples of efficient transcription of 'tissue-specific' genes in oocytes have now been documented (eg. human zeta globin - Proudfoot et al., 1984; the chicken histone H5 gene - Wigley et al., 1985 and this thesis). Differential transcription of genes from different species and with distinct patterns of expression is likely to reflect the presence or absence of endogenous oocyte transcription factors able to interact with promoter regions.

Some of the most detailed analyses of transcriptional promoter elements have been carried out using Xenopus oocytes. The two major examples are the work by Birnstiel's group on sea urchin histone genes (eg. Grosschedl et al., 1983) and the work by McKnight on the HSV TK gene (eg. McKnight et al., 1984).

(4) Co-injection experiments

Much of the experimental work presented in this thesis involves the co-injection of DNA with other molecules (cell extract components) into

Xenopus oocytes. The approach taken is based upon an idea by Gurdon and Melton (1981) for the use of the oocyte as an assay to identify regulatory factors. Since 1981, several studies have utilised oocyte co-injection for this and other purposes, and a brief review of some of this work follows.

Experiments with sea urchin histone genes showed that transcription of the H3 gene was correctly initiated in frog oocytes, but readthrough occurred at the 3' end (Hentschel et al., 1980). Co-injection of a salt-wash fraction from sea urchin embryo chromatin, with H3 gene-containing DNA, resulted in the formation of correct H3 mRNA 3' ends (Stunnenberg and Birnstiel, 1982). Although it originally appeared likely that the regulatory factor responsible for this effect was a protein, subsequent experiments demonstrated that the active component was a short (about 60 nucleotides) RNA molecule (Galli et al., 1983). The original factor was therefore suggested to be a small nuclear RNP. Interestingly, purified RNA was able to cause the appearance of correct H3 3' ends after co-injection, suggesting that oocyte proteins could substitute for sea urchin proteins in the formation of an active RNP complex. Finally, sequence analysis of cDNA clones representing the active RNA molecule, termed U7 RNA, revealed that extensive complementarity existed between this RNA and conserved sequences at the 3' end of histone mRNAs (Strub et al., 1984). Using this information, models for histone mRNA 3' end processing have been proposed (Strub et al., 1984; Birnstiel et al., 1985).

In recent work, Birnstiel's group have used the oocyte co-injection assay to demonstrate specific stimulation of sea urchin H2B gene transcription by a chromatin protein fraction (Mous et al., 1985). As noted in section 1-4-4(3), two sequence regions of the H2B gene are able to mediate the stimulation effect and both of these are located downstream

from the cap site. Although not discussed by Mous et al. (1985), it is puzzling that H2B stimulation was not observed in the original experiments by Stunnenberg and Birnstiel (1982), since the chromatin salt wash fractions were prepared by identical procedures and from the same source in both sets of experiments.

In their studies on chromatin assembly and gene expression in frog oocytes, Worcel's group have also made extensive use of the co-injection technique. The major findings from these studies have already been presented in section 1-2-3.

Finally, Jones et al. (1983) demonstrated that E1A gene-containing plasmids, or a cell extract containing E1A proteins, gave rise to stimulation of transcription from the adenovirus E3 promoter after co-injection into oocytes. Subsequent experiments have utilized oocyte co-injection to define a domain of the E1A protein sufficient for this stimulatory effect (Richter et al., 1985; see section 1-4-4(2)).

1-6 Histone H5

Research on chicken histone H5, at both the protein and nucleic acid levels, has been a central concern of this laboratory for many years. H5 is also the focus of the work presented in this thesis. The following is a brief discussion of histone H5 with some general background information on histone proteins and the chicken histone gene family.

1-6-1 Histone Proteins

The histones consist of five classes of small, basic proteins, viz., the core histones: H2A, H2B, H3, H4, and the linker histone H1, and their variants (Isenberg, 1979; Von Holt et al., 1979). The core histones are involved in the formation of the nucleosome (Richmond et al., 1984), and linker histones bind at the exit and entry points of DNA from the nucleosome core and are believed to be involved in the generation of higher

order chromatin structures (Allan et al., 1980; Igo-Kemenes et al., 1982).

Although histones are generally well conserved throughout evolution, variants exist for all histone types. Firstly, various post-translational modifications can occur with histones, such as acetylation and phosphorylation (Isenberg, 1979). Secondly, within a single organism, there exist non-allelic, primary structure variants (subtypes) of H1, H2A, H2B and H3 (Isenberg, 1979; Von Holt et al., 1979; Zweidler, 1984). These variants can be expressed differentially during development, the cell cycle and the differentiation of specific cell types. It should also be noted that some specific histone variants have been highly conserved through evolution (Zweidler, 1980).

With regard to cell cycle regulation, histone subtypes have been classified into three groups (Zweidler, 1984). These are: replication-dependent subtypes (expressed strictly during DNA synthesis i.e., S phase), partially replication-dependent subtypes (induced at the start of S phase, but not completely repressed at its end) and replication-independent subtypes (expressed throughout the cell cycle; also referred to as basal histones).

In the chicken, variants of histones H1, H2A, H2B and H3 have been characterised. Urban and Zweidler (1983) investigated H2A, H2B and H3 subtypes and found that the proportions of proteins within the three classes of subtypes change independently throughout chicken embryonic development. Also, different relative amounts of variants are found in different adult tissues. Similarly, the ratio of different chicken H1 subtypes has been shown to vary between tissues and during the differentiation of particular cell lines (Berdikov et al., 1975; Winter et al., 1985).

Tissue-specific histone variants have also been identified. Aside from histone H5 (see below), most of these variants are sperm-specific. Examples of sperm-specific subtypes have been characterised for both core histones (Zweidler, 1984) and histone H1 (Cole et al., 1984).

Finally, the occurrence of histone variants, their conservation throughout evolution and their differential regulation during development and in different tissues, suggests a possible role for these proteins in the regulation of cellular processes, such as replication and transcription.

1-6-2 H5 Protein

H5 is an extreme linker histone variant, found only in the nucleated erythroid cells of some non-mammalian vertebrates (Neelin et al., 1964; Aviles et al., 1978; see below). H5 has both sequence and structural similarities with H1 (Yaguchi et al., 1977; Von Holt et al., 1979; Aviles et al., 1978) and shows particularly strong similarities with another linker histone variant, $H1^{o}$ (Smith et al., 1980; Cary et al., 1981). This latter protein is a mammalian histone which is found in many tissues, and only appears once cells have terminated their maturation (Gjerset et al., 1982).

In the chicken, immunological studies have shown that H5 protein is present only in erythroid cells (Shannon et al., 1985 - this laboratory). H5-like proteins have also been identified in erythrocytes from several other birds (Neelin, 1968), from a range of different fish (Miki and Neelin, 1977; Goetz et al., 1978), from amphibia (Destree et al., 1979) and from reptiles (Tsai and Hnilica, 1975). However, it remains controversial as to whether or not these latter cases, particularly the non-avian examples, represent the identification of true, erythroid-specific H5 proteins. In fact, it is now apparent that amphibia and reptiles have an

$H1^{o}$-like protein, which is present in many tissues (Rutledge et al., 1984; Smith et al., 1984; Moorman and de Boer, 1985). In general, then, all vertebrates appear to possess an $H1^{o}$/H5-like histone, but the distribution of this protein is dramatically different in the various classes of organisms.

Levels of H5 protein increase during maturation of avian erythroid cells (Moss et al., 1973; Weintraub, 1978). This increase is correlated with several changes that occur within the cells, such as chromatin condensation, an increase in the nucleosome repeat length and the shutdown of replication and transcription (Neelin et al., 1964; Ruiz-Carrillo et al., 1974; Weintraub, 1978). As the erythroid cells mature, H5 partially, but not completely, replaces H1 molecules on chromatin (Torres-Martinez and Ruiz-Carrillo, 1982; Mazen et al., 1982). Bates and Thomas (1981) have determined that in chicken erythrocytes approximately 0.9 molecules of H5 are present per nucleosome compared with about 0.4 molecules of H1. Immunological and chemical cross-linking studies suggest that H5- and H1-associated nucleosomes are interspersed, perhaps randomly, in chromatin (Torres-Martinez and Ruiz-Carrillo, 1982; Mazen et al., 1982; Lennard and Thomas, 1985).

Work by several groups has elucidated some of the functional properties of histone H5. H1 and H5 have been found to occupy equivalent sites in chromatin, but H5 has a greater chromatin binding affinity (Kumar and Walker, 1980). Accordingly, H5 redistributes itself among binding sites less readily than H1 (Caron and Thomas, 1981). Thomas et al. (1985) found that H5 promotes the association of condensed chromatin fragments in vitro and this results in the formation of pseudo-higher-order structures. This property seems to be unique to H5, as compared with standard H1

proteins. Previously, competition experiments had suggested a slightly greater preference of H5, relative to H1 for higher-order structures (Thomas and Rees, 1983). All of the above observations suggest a crucial role for histone H5 protein in generating the enhanced stability of the higher-order structures in chicken erythrocyte chromatin (Thomas et al., 1985).

These results are also consistent with a role for H5 in the generation and/or maintenance of the synthetically inactive state of the red cell nucleus. It should be noted, however, that the presence of H5 protein per se is not sufficient for the generation of an inactive state, since H5 is present in transcriptionally active, dividing erythroblasts (Appels et al., 1972). It appears likely that increased levels of H5 protein are responsible, perhaps in concert with other factors, for the inactive state of the mature avian erythrocyte nucleus (Bates and Thomas, 1981).

1-6-3 Histone Genes

Histone genes have been the focus of a great deal of research, as outlined in several recent reviews (eg. Hentschel and Birnstiel, 1981; Maxson et al., 1983; Old and Woodland, 1984; 'Histone Genes': Stein, Stein and Marzluff, eds., 1984). It has been found that there are two general types of histone gene organisation - tandemly repeated genes (such as sea urchin 'early' genes) and clustered but disordered arrangements (such as the histone genes of the chicken - see below). Individual gene copy numbers range from 2 in yeast up to many hundreds in other organisms. The majority of histone genes studied contain no introns and are transcribed into non-polyadenylated mRNAs. Also, the S phase-linked expression of histone proteins (see 1-6-1) has been found in many cases to be regulated at the mRNA level, involving control of both transcription and mRNA stability.

Molecular analyses, particularly those of Birnstiel's group, have elucidated some of the requirements for histone gene expression. Sequences important for efficient and accurate transcription of histone genes have been identified (eg. the sea urchin H2A gene - Grosschedl and Birnstiel, 1980; Grosschedl et al., 1983). The highly conserved dyad symmetry element of the 3' end of most histone mRNAs, and sequences further downstream, have been found to be necessary for 3' end formation (Birchmeier et al., 1983). Moreover, correct 3' ends are generated by post-transcriptional processing (Krieg and Melton, 1984) and this reaction involves a specific small nuclear RNP (Birchmeier et al., 1984; see 1-5-2 (4)). Other trans-acting factors involved in histone gene expression have also been identified (see 1-4-4(3) and 1-5-2 (4)).

This laboratory has isolated and mapped most, if not all, of the histone genes of the chicken, in a number of lambda and cosmid clones (D'Andrea et al., 1985 and references cited therein). Each major gene type is represented 6 to 10 times. The genes have a clustered but generally disordered arrangement, although there are some preferred associations (D'Andrea et al., 1985).

'Variant' chicken histone genes have also been characterised in this and other laboratories. These genes are isolated and are not closely linked to other histone genes. They differ markedly in their structure and expression from 'standard' histone genes and encode variant histone proteins. Four such genes have been isolated to date; two of these encode the H3.3 protein (Brush et al., 1985), one codes for the H2A.F protein (= H2A.Z = M1; Harvey et al., 1983; A. Robins - personal communication), and the other encodes histone H5 (Krieg et al., 1983; see below). Transcripts from all of these genes are polyadenylated and the genes for

H3.3 and H2A.F contain introns (Brush et al., 1985; A. Robins - personal communication). Also, the expression of each gene is replication-independent (Dalton et al., 1986a; S. Dalton - personal communication; Brush et al., 1985). The presence of introns and poly A tails is likely to be related to this S phase-independence (Old and Woodland, 1984).

This laboratory has characterised sequence elements involved in the transcription of several chicken histone genes. Work has focused on an H1 gene (Younghusband et al., 1986), an H2A/H2B divergently transcribed gene pair (Sturm et al., 1986) and the H2A.F gene (R. Sturm - personal communication). Common elements such as GC boxes and CAAT boxes were found to be important for efficient histone transcription (see 1-3-4(2)). Two gene-specific upstream elements have also been identified, for H2B genes (Harvey et al., 1982) and H1 genes (Coles and Wells, 1985). These motifs are conserved among the appropriate histone genes of many different organisms, but as yet their function remains unknown.

1-6-4 The Chicken H5 Gene

Chicken H5 cDNA and genomic clones were first isolated in this laboratory (Krieg et al., 1982a; Krieg et al., 1982b; Krieg et al., 1983). Ruiz-Carrillo and his co-workers have also reported the isolation of cDNA and genomic clones for chicken H5 (Ruiz-Vazquez and Ruiz-Carrillo, 1982; Ruiz-Carrillo et al., 1983) and Doenecke and Tonjes (1984) have characterised the duck H5 gene.

As mentioned above, the single-copy chicken H5 gene is not closely linked to other, core or H1, histone genes (Krieg et al., 1983; Ruiz-Carrillo et al., 1983). The complete sequence of the transcribed portion of this gene and large regions of flanking sequence have been determined (Krieg et al., 1983; Ruiz-Carrillo et al., 1983). Sequence comparisons with chicken H1 genes suggest that the H5 gene may have evolved from an

ancestral H1 gene (Coles and Wells, 1985).

Chicken H5 mRNA is polyadenylated (Molgaard et al., 1980; Krieg et al., 1982b) and transcription of the H5 gene is replication-independent (Dalton et al., 1986; see 1-6-3). Krieg et al. (1982b and 1983) predicted the 5' and 3' ends of chicken H5 mRNA from sequence data. Work presented in this thesis tests these assignments using primer extension and S1 nuclease analyses (see chapter 2 and Wigley et al., 1985).

The 5' flanking region of the chicken H5 gene includes the sequence CTTAAAT, identified as the likely TATA box of this gene (Krieg et al., 1983). The H1 gene-specific element is not present in the upstream region of the H5 gene (Coles and Wells, 1985; see 1-6-3). The 3' flanking region of the gene contains neither an AATAAA motif nor the conserved histone dyad symmetry element (Krieg et al., 1982b). However, another sequence capable of forming a hairpin loop structure is found just upstream from the polyadenylation site in an H5 cDNA clone (Krieg et al., 1982b). A similar element is found in the 3' flanking region of the chicken H2A.F gene (A. Robins - personal communication).

Ruiz-Carrillo (1984) identified S1 hypersensitive sites in the 5' and 3' flanking regions of the chicken H5 gene, when the gene was present on supercoiled plasmids. The H5 gene also exhibits several DNase I hypersensitive sites in the chromatin of cells expressing the gene (Bergman, 1986). Furthermore, in different erythroid cell types, the degree of DNase I hypersensitivity of the H5 gene correlates with the level of H5 transcripts present in these cells. Finally, studies in this laboratory suggest that the chicken H5 gene is preferentially associated with the nuclear matrix in an erythroid cell line expressing the gene, but not in a T cell line in which the gene is not expressed (Dalton et al., 1986b).

1-7 Aims of this Project

Work in this laboratory has focused on the histone gene family of the chicken, with particular emphasis on the gene for the unique histone variant H5. As described in the preceding section (1-6-4), the single-copy chicken H5 gene has been isolated and sequenced in this laboratory and its relationship to other chicken histone genes has been determined. The primary aim of the work presented in this thesis was to investigate the control of transcription of the H5 gene, particularly at the level of regulation by trans-acting factors. The Xenopus oocyte system was chosen for use in this study because it had already been shown to be applicable for identifying regulatory factors involved in gene expression (see 1-5-2(4)).

The initial aim of this work was to investigate chicken H5 gene transcription in injected oocytes. Following this, the primary aim was to use oocyte co-injection to identify trans-acting factors, from chicken erythroid cells, able to regulate transcription of the H5 gene. If successful, preliminary characterisation of the nature of the trans-acting factors and their effect on the H5 gene would be carried out.

A secondary aim of the work described in this thesis was to utilise the oocyte system to investigate the possible effect of the chicken H5 protein on transcription of co-injected genes.

CHAPTER 2

EXPRESSION OF THE CHICKEN HISTONE H5 GENE IN XENOPUS OOCYTES

CHAPTER 2 : EXPRESSION OF THE CHICKEN HISTONE H5 GENE IN XENOPUS OOCYTES

2-1 Introduction

In order to identify regulatory factors involved in the transcription of the chicken H5 gene, it was first necessary to test for the expression of the gene in a suitable system. The Xenopus oocyte was chosen as the assay system because of the advantages noted in 1-5-2 and because it had been used successfully, in other studies, for the identification of regulatory factors (1-5-2(4)).

The chicken H5 gene had previously been isolated and sequenced in this laboratory (Krieg et al., 1983). The initial phase of my work involved establishing expression of this gene in frog oocytes and determining some of the parameters of H5 gene expression in vivo and in injected oocytes. Some of the work described in this chapter has been published previously (Wigley et al.,1985).

2-2 Expression of the H5 gene: 5' end mapping of H5 transcripts from chicken erythroid cells and injected oocytes

The H5 gene used in these studies had been subcloned from a lambda clone (Krieg et al., 1983) into pBR322 and the resulting construct is named pH52.6 (gift from J.A. Whiting). This contains approximately 2.6 kilobase pairs (kb) of chicken DNA, which includes the entire transcribed region of the H5 gene (875 base pairs (bp)), about 1200 bp of 5' flanking region and about 500 bp of 3' flanking region (figure 2.1(a)). The sequence from -784 to +1059 (relative to the cap site at +1) has previously been determined (Krieg et al., 1983).

For expression studies, approximately 5 ng of pH52.6 was injected into the nucleus of each of a batch of 20 to 30 oocytes. (9-3-14; the first H5 gene injections, only, were performed by Dr. Rick Sturm.) Total oocyte RNA was isolated (9-3-17) and transcription analysis was carried out by primer

Figure 2.1 : The chicken H5 gene and flanking sequences

(a) A schematic representation of the chicken histone H5 gene is shown. The boxed area represents the transcribed region of the gene. The numbers are base pairs (bp); +1 indicates the start site of transcription (cap site). The square brackets represent the region of the gene for which sequence data have previously been obtained (from -784 to +1059; Krieg et al., 1983).

5' U/T = 5' untranslated region

3' U/T = 3' untranslated region

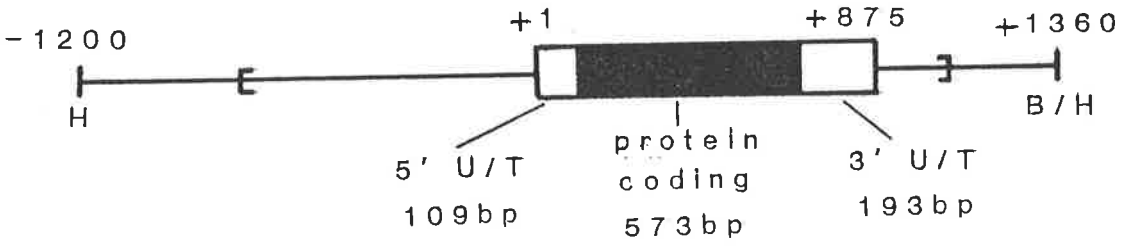
H = HindIII restriction site

B = BamHI restriction site

(b) The sequence of the H5 gene from -256 to the ATG initiation codon is shown. The cap site designated in this chapter is indicated with an arrow (which represents the endpoint of the longer primer extension product shown in figure 2.2). The numbers above the sequence indicate the distance in bp from the cap site; the exact base associated with the number is directly beneath the second digit from the right (or directly beneath the 1 in the case of the cap site). The binding site for the H5 26-mer is also indicated. The two putative GC boxes (see chapter 5) and the 'TATA' box are shown spaced away from the rest of the sequence.

(c) The sequence of the H5 gene from the TGA stop codon to +1059 is shown. Numbering is as described in (b). The 3' ends of the H5 transcripts in AEV cell RNA and RNA from injected oocytes are indicated with arrows. The SmaI and FnuDII sites used to prepare the probe for 3' S1 nuclease assays are shown. The arrows above the sequence around +850 represent a region of dyad symmetry, which may form a hairpin-loop structure in the RNA (Krieg et al., 1982b).

(a) chicken H5 gene



(b) 5' sequence

-250 -200

CCCTTGTCCCCACCATGTCCATCCCATTCCCACCGTGTCTCACCCTATCCCCACCGTATCCCTCTCCATCCCTTA

-150

ATGTCCAACCCCAGCGT CCGCCC CTTTCCCCATCACATCCCTTCTGGTCCCAACCTCGTCCCTCCCTGCCTGC

-100 -50

CCCAGGCATGTCCTGCG GGGCGG GGCAGAGGGGGGACACGGGGACAGGCAGTCCTCCCCGCCGTCCGTGCCGC

+1

ACCC TTAAAT GCGTGCTGGTGGCGACGCGCGGCCGCAGACGCAGCGGCGGCGGCAGCAGGAGCAGCCCCACAT

+50 +100

CCGTTGTTGCTGGCGGCTCCTTTTTTAAGCTCCCTAACCCCAGTGCCCTGCCGTGGGGTGAAGCGGCGGCC ATG

26-mer

(c) 3' sequence

Sma I +700 +750

TGA GCAGCCCCGGGGCTTTGCCCAGGCTCTCCCCATTGGTTTCTGTAAATAGCTTTTGCCTTTATTTTTTACCTCT

+800

TTCTATTTGCAAATTTTATAAGTTGATCTATTCCTAAGAGCTAAAACAAGGCAACGAATGAAAGAAAAAAAGAAA

+850 in vivo +900

CAAAAATGGAACTTCTTCCATATGGAAGAGTTCCCGTTTATAAAAGC AACTTCTCTGAGTGTTTATTTCATCTT

oocyte

+950

GCCGTGGGTCTGGAGGGTTGCGGTGGGCGGTGGAGGAGCTTGGGGGGAGGTAGGAGGGCTGTGAGAGCCAGGAGG

+1000 FnuDII +1050

AGGAGAGGGGACTCCTCCTTGTCCATAGGAGTGAGGCACAGCCCGCCAGCCCAGCGCGTGAGGGCTGGAGATAAC

AGTGCGG

extension (9-3-21) with an H5 gene-specific 26 base primer (9-3-12; figure 2.1(b)). The RNA equivalent to that from 1 or 2 oocytes was commonly used in this type of analysis.

(1) 5' end mapping with RNA from injected oocytes

Figure 2.2, track 1, shows the result of primer extension on RNA from oocytes injected with pH52.6. Two major bands are visible, the upper band representing an extension product of approximately 111 bases. [Note that no bands are produced when extension is carried out with RNA from uninjected oocytes (data not shown).]

This result shows that the chicken H5 gene is transcribed in frog oocytes and the longer extension product maps the 5' end of the H5 mRNA to the position indicated in figure 2.1(b). This corresponds to the transcription start site predicted by Krieg et al. (1983), which is located about 30 bp downstream from the likely TATA box of the H5 gene (figure 2.1(b)). The shorter extension product may represent the use of a second start site or may result from premature termination of reverse transcription (McKnight et al., 1981).

Subsequent injection experiments showed that the H5 gene is transcribed at a similar level to a chicken H1 gene and at a much lower level than chicken H2A and H2B genes (Sturm et al., 1986) and the chicken ALA-synthase gene (Maguire et al., 1986).

(2) 5' end mapping with RNA from chicken erythroid cells

To determine the 5' end of chicken H5 mRNA, total cytoplasmic RNA was isolated from cultured, AEV-transformed, chicken erythroid cells (termed 'AEV cells' in this thesis; 9-3-15, 9-3-17; Beug et al., 1982). These cells are blocked at a pre-erythroblast stage of differentiation (Samarut and Gazzolo, 1982) and contain H5 protein (Beug et al., 1979). Primer extension

was carried out on the AEV cell RNA, with the H5 26-mer, and the result is shown in figure 2.2, track 2. As with the oocyte result, two major bands are visible. However, the longer extension product is 120 bases in length, which is 9 bases longer than the corresponding extension product for injected-oocyte RNA. This is likely to be due to the presence of a 9 base insertion in the 5' untranslated region of the AEV cell H5 mRNA, compared with the transcript from the injected H5 gene. This polymorphism has previously been observed in a chicken H5 cDNA clone (Ruiz-Vazquez and Ruiz-Carrillo, 1982). Allowing for this difference, the 5' end of the AEV cell H5 mRNA maps at the same point as the injected-oocyte transcript. This result has also been obtained using RNA from chicken reticulocytes.

The cap site of H5 mRNA that I designate here differs from that designated by Ruiz-Carrillo et al. (1983). As previously suggested (Wigley et al., 1985), although the published primer extension data appear to be accurate, it appears that several arithmetical and interpretative errors were made in deducing the H5 cap site from these data. Specifically :

(i) The Sau3AI - AluI restriction fragment used as a primer by Ruiz-Carrillo et al. had its 5' end at +99, and not at +95 as indicated in their paper;

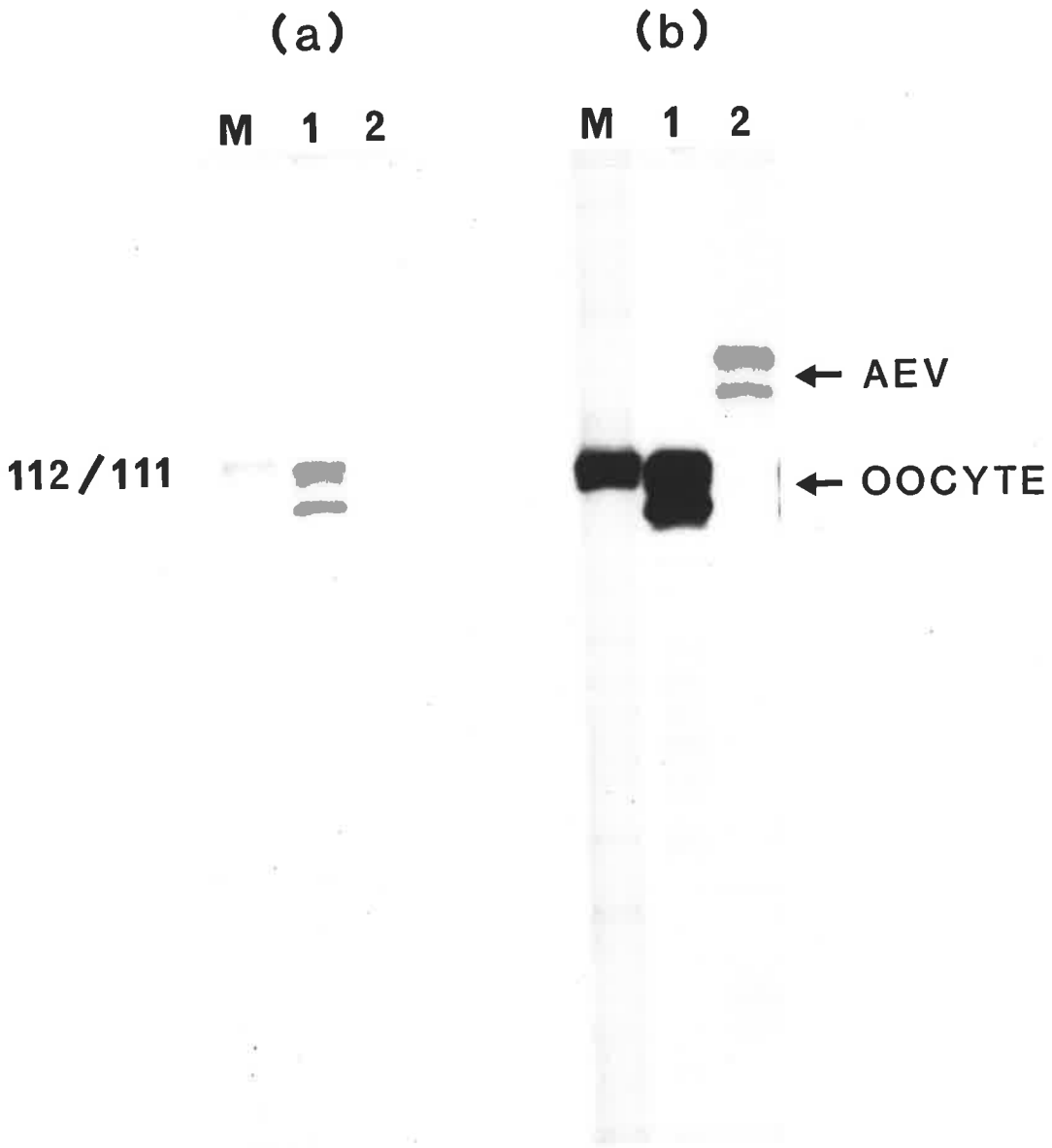
(ii) It was stated that reverse transcriptase fails to copy the 5'-terminal and penultimate nucleotides of capped mRNA. The reference given for this information is concerned with sequencing of viral RNA by reverse transcription (Akusjarvi and Petterson, 1979). These authors actually state that their reverse transcribed copies were extended to the extreme 5' end of the mRNA used, but that the final two nucleotides did not resolve on their gels;

(iii) A correction of 10 bases was made for the polymorphic insertion/deletion of 9 bases.

Figure 2.2 : 5' end mapping of H5 transcripts from AEV cells and injected oocytes

The 5' ends of H5 transcripts in RNA from AEV cells and from oocytes injected with the H5 gene were mapped by primer extension with the H5 26-mer. Extension products and markers were denatured by heating at 100 ^{o}C in formamide loading buffer and were electrophoresed on a 6% polyacrylamide sequencing gel. Two exposures of the same autoradiograph are shown ((a) and (b)).

Track M is a marker track, in which end-labelled HpaII-cut pUC19 DNA (obtained from Biotechnology Research Enterprises of South Australia [BRESA]) was run; the band shown is a doublet of 112 and 111 bp. Track 1 shows the result obtained with RNA from injected oocytes; an amount equivalent to the RNA from one oocyte was used. A large number of experiments indicate that the longer extension product is 111 bases in length. Track 2 shows the result obtained with 10 ug of total RNA from AEV cells; the longer extension product is 120 bases in length.


(a)
(b)
M 1 2
M 1 2
AEV
112/111
OOCYTE

2-3 3' end mapping of H5 transcripts from chicken erythroid cells and injected oocytes

To further characterise the fidelity of H5 gene expression in Xenopus oocytes, RNA from AEV cells and injected oocytes was subjected to 3' end analysis using the S1 nuclease technique.

A 344 bp SmaI-FnuDII restriction fragment was used in this analysis (figure 2.1(c)). This was labelled using T4 DNA polymerase (9-3-20) or by extension on an M13 subclone (9-3-20). Both double-stranded and single-stranded probes were used (9-3-20), although the latter were preferred, since they gave consistently better results.

(1) 3' end mapping with RNA from chicken erythroid cells

The result of 3' S1 nuclease analysis on poly A$^+$ RNA from AEV cells is shown in figure 2.3(a), track 2. Two major bands are observed, at approximately 186 bases and 175 bases. The same result was also obtained with chicken reticulocyte RNA.

The larger S1 product maps the 3' end of H5 mRNA at the polyadenylation site, previously determined by comparison between an H5 cDNA sequence and the genomal sequence (Krieg et al., 1982b and 1983). This site follows a large, potential stem-loop structure in the H5 mRNA (Krieg et al., 1982b; figure 2.1(c)).

The shorter S1 product maps the 3' end of H5 mRNA approximately 11 bases upstream from the previously designated polyadenylation site (figure 2.1(c)). This result could be due to the presence of two populations of H5 transcripts or could be an artifact of the S1 nuclease technique. Several observations suggest that the former explanation is correct :

(i) 3' S1 nuclease analysis using a different restriction fragment also generated two products, differing in size by approximately 11 bases;

(ii) Changing the temperature of hybridisation in the mapping protocol did not alter the result;

(iii) Two products, differing in size by about 11 bases, were also obtained when the 3' end of duck H5 mRNA was mapped using S1 nuclease analysis (Doenecke and Tonjes, 1984);

(iv) Only the shorter S1 product was obtained with RNA from oocytes injected with the H5 gene (figure 2.3(a), tracks 1 and 3; see discussion below).

(2) 3' end mapping with RNA from injected oocytes

Figure 2.3(a), tracks 1 and 3, show the result of 3' S1 nuclease analysis on RNA from oocytes injected with pH52.6. As mentioned above, only one major product is observed, which co-migrates with the shorter product obtained with AEV cell RNA. This suggests that two different 3' ends of H5 mRNA are generated in chicken cells and that only one of these 3' ends is produced in injected oocytes.

The oocyte result also argues against the possibility that the two chicken H5 mRNA 3' ends are derived from the two alleles of the H5 gene. The sequences of the 3' end of the gene injected into oocytes and the H5 cDNA clone are identical (except for single base changes) up to the polyadenylation site and, therefore, one would expect the longer S1 product to be generated with injected-oocyte RNA; however, it is the shorter product which is observed.

2-4 Are chicken H5 transcripts polyadenylated in frog oocytes ?

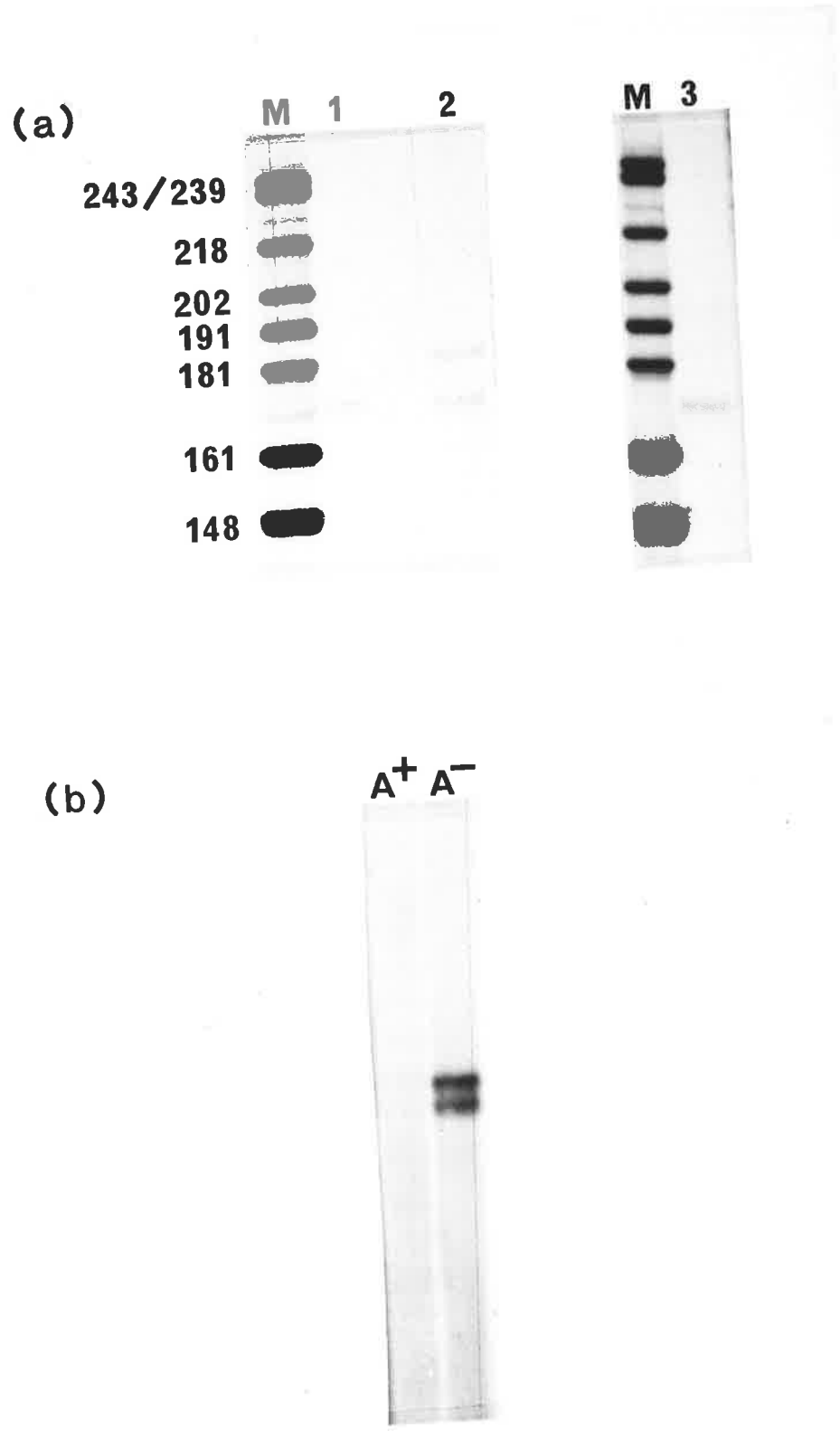
H5 mRNA is polyadenylated in vivo (Molgaard et al., 1980; Krieg et al., 1982b). To determine whether transcripts from injected H5 genes were polyadenylated in oocytes, RNA from pH52.6-injected oocytes was separated into poly A^+ and poly A^- fractions on a poly U-sepharose column (9-3-18).

The separation was checked by primer extension on RNA from both

Figure 2.3 : 3' end mapping of H5 transcripts

(a) The 3' ends of H5 transcripts in RNA from AEV cells and oocytes injected with the H5 gene were mapped by S1 nuclease analysis. The SmaI-FnuDII fragment indicated in figure 2.1(c) was used in this analysis. Track M is a marker track, in which end-labelled HpaII-cut pBR322 DNA was run; the sizes of the fragments (in bp) are shown. Tracks 1 and 3 show two independent results obtained with RNA from injected oocytes (an amount equivalent to the RNA from two oocytes); the result is best seen in track 3. One major band is observed; the results of a number of experiments indicate that the size of this band is 175 bases. Track 2 shows the result obtained with 1 ug of poly A^+ AEV cell RNA. Two major bands are seen; these routinely map at 186 and 175 bases.

(b) Using poly U-sepharose, RNA from oocytes injected with the H5 gene was separated into poly A^+ and poly A^- fractions. Samples of these, equivalent to the RNA from two oocytes, were analysed by primer extension with the H5 26-mer. It is clear that the majority (if not all) of the H5 transcripts are found in the poly A^- fraction.


(a)
M
1
2
M
3
243/239
218
202
191
181
161
148
(b)
A+
A−

fractions with an 11-mer, originally used to isolate an H5 cDNA clone (Krieg et al., 1982a). This 11-mer hybridises with a number of oocyte transcripts, resulting in a ladder of extension products. It was found that these products were distributed differentially in the A^+ and A^- fractions, indicating that (at least some) separation had been achieved.

RNA from both fractions was then analysed by primer extension with the H5-specific 26-mer and the result is shown in figure 2.3(b). This demonstrates that most, if not all, chicken H5 transcripts are not polyadenylated in frog oocytes, in contrast to the situation in chicken red cells ~~vivo~~. This is discussed further below (2-6).

2-5 Cell-type specificity of H5 mRNA in vivo

To further characterise the parameters of H5 gene expression in vivo, the cell-type specificity of H5 mRNA was investigated. This was also relevant to the aim of identifying transcription factors for the H5 gene, since factors may exist which are involved in the erythroid cell-specific transcription of the H5 gene.

Total RNA was isolated from AEV cells and from two other transformed chicken cell lines: a fibroblast line and a T cell line (9-3-15). After denaturation with formamide and glyoxal (9-3-19), approximately 15 ug of each RNA sample was electrophoresed on a 1.5% agarose gel and transcripts were analysed by Northern hybridisation (9-3-19) with a nick-translated chicken H2B gene fragment (9-3-13; Sturm et al., 1986) and the 2.6 kb H5 gene insert from pH52.6, labelled by the random priming method (Feinberg and Vogelstein, 1983). The result is shown in figure 2.4 (and has previously been published in Shannon et al., 1985).

As expected, H2B mRNA is found in all three cell types. In contrast, H5 transcripts are only found in the RNA isolated from the erythroid cell

Figure 2.4 : Cell-type specificity of H5 mRNA

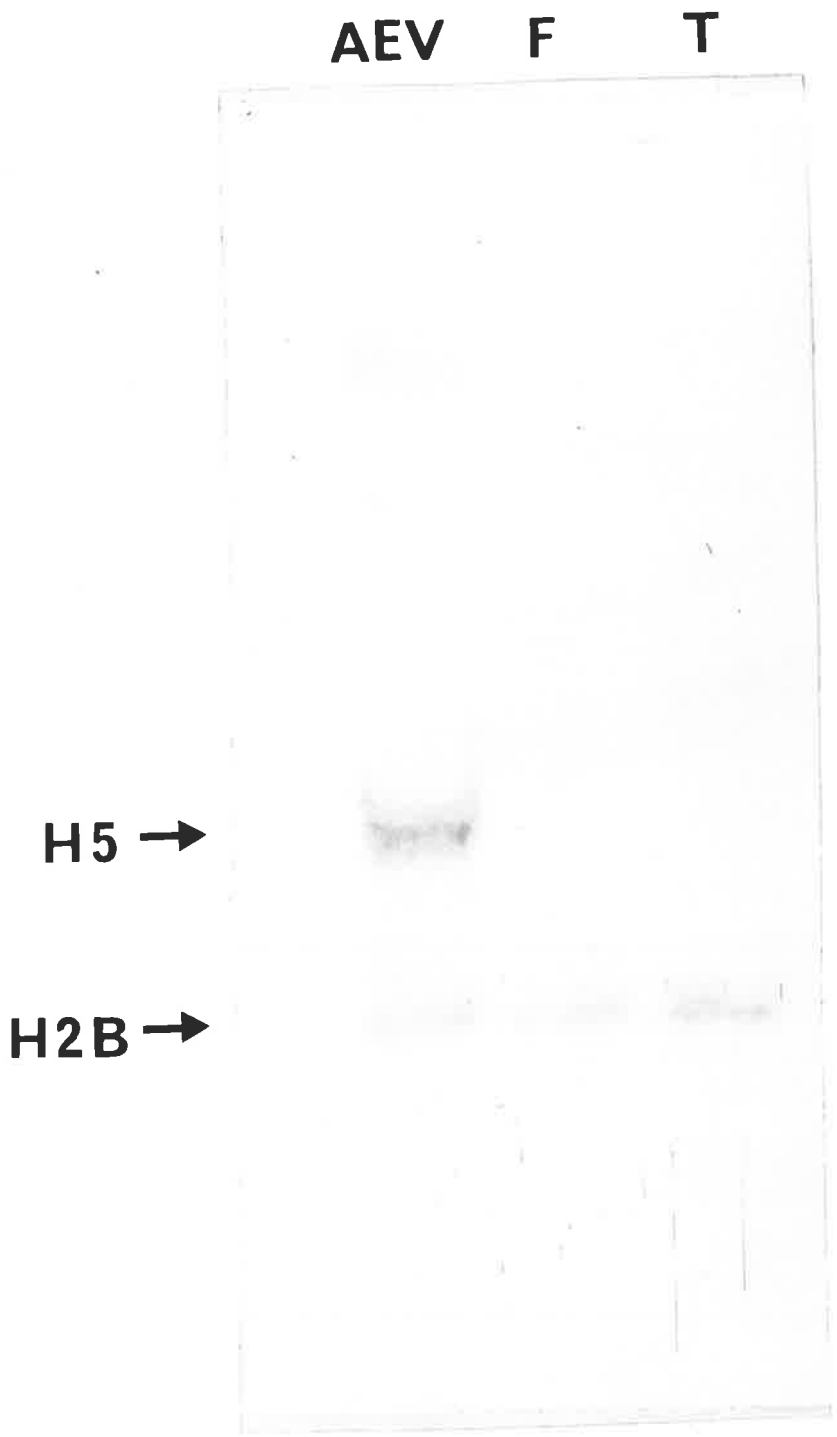
The result of Northern hybridisation analysis of H5 and H2B mRNA is shown. Approximately 15 ug of RNA from each of three different chicken cell lines was electrophoresed on a 1.5% agarose gel, following denaturation with formamide and glyoxal. After transfer, the nitrocellulose filter was hybridised simultaneously with H5 and H2B probes.

AEV = RNA from AEV cells, an erythroid cell line

F = RNA from a fibroblast cell line

T = RNA from a T lymphocyte cell line

The bands representing H5 and H2B mRNA are indicated.


AEV
F
T
H5
H2B

line. This indicates that the erythroid cell-specificity of H5 protein (Shannon et al., 1985) is regulated at the level of mRNA availability. This, in turn, may be controlled at the level of H5 gene transcription or H5 mRNA stability. Following precedents in other systems (refer to chapter 1), the former may be the more likely level at which regulation occurs.

2-6 Discussion

The 5' end mapping experiments described in this chapter show that transcription of the chicken H5 gene is accurately initiated in Xenopus oocytes. Furthermore, transcription from the H5 promoter occurs at a low, but significant, level.

This is one of the few examples of a gene which is expressed in a cell type-specific manner being transcribed with fidelity in frog oocytes. The human zeta-globin gene is also transcribed efficiently in oocytes (Proudfoot et al., 1984), but other 'cell type-specific' genes, such as chicken ovalbumin and rabbit beta-globin, are not (Gurdon and Melton, 1981). The ability of the H5 gene to be transcribed in oocytes may be related to the presence of two GC boxes (1-3-4(2), 1-4-4(3)) in the 5' flanking region of the gene (figure 2.1(a); also refer to chapter 5). The GC boxes of the HSV TK gene are known to be important for the efficient transcription of this gene in frog oocytes (McKnight et al., 1984).

From 3' end mapping experiments it appears likely that there are two populations of H5 transcripts in chicken erythroid cells, differing in the location of their polyadenylation site by approximately 11 bases. In oocytes injected with the H5 gene only one of these sites is selected as the mature 3' end. Furthermore, in contrast to the situation in vivo, H5 transcripts are not polyadenylated in injected oocytes.

H5 mRNA contains neither an AAUAAA-like sequence at the appropriate distance upstream from the mature 3' end(s), nor the dyad symmetry element

involved in the generation of other histone mRNA 3' ends (Krieg et al., 1982b; Birchmeier et al., 1983). Both of these elements are known to be involved in the processing of precursors to form mature mRNA 3' ends (Wickens and Stephenson, 1984; Krieg and Melton, 1984). It is possible that the large potential stem-loop structure found near the end of the mature H5 mRNA is involved in a processing reaction to form the H5 mRNA 3' end. The partial nature of H5 mRNA 3' end formation and the lack of polyadenylation observed with transcripts derived from the H5 gene injected into oocytes may be due to an inability of the oocyte to recognise the appropriate regulatory signals in the H5 gene sequence. This in turn may reflect an absence of the appropriate factors in oocyte nuclei.

[Note that although the sequence UAUAAA is found near the 3' end of H5 mRNA, it is located between the two 3' ends found for chicken H5 mRNA and is absent from the duck H5 mRNA 3' untranslated region (Doenecke and Tonjes, 1984). However, a similar potential stem-loop structure is found at the same position in both the duck and chicken sequences (Doenecke and Tonjes, 1984).]

The primary aim of this work was to identify regulatory factors involved in the initiation of transcription of the H5 gene. The finding that transcription of the H5 gene was accurately initiated in Xenopus oocytes and that this transcription was at a low, but easily detectable, level, suggested that the frog oocyte was a suitable system with which to approach this aim.

CHAPTER 3

EFFECT OF CHROMATIN SALT-WASH FRACTIONS FROM CHICKEN ERYTHROID CELLS ON H5 TRANSCRIPT LEVELS IN CO-INJECTED OOCYTES

CHAPTER 3 : EFFECT OF CHROMATIN SALT-WASH FRACTIONS FROM CHICKEN ERYTHROID CELLS ON H5 TRANSCRIPT LEVELS IN CO-INJECTED OOCYTES

3-1 Introduction

To identify factors involved in the transcription of the H5 gene, I modified the approach taken by Stunnenberg and Birnstiel (1982) in their work on the factors required for correct formation of sea urchin H3 mRNA 3' ends in frog oocytes. This involved:

(i) Isolating salt-wash fractions from chicken erythroid cell chromatin,

(ii) Co-injecting these fractions with the H5 gene into Xenopus oocytes, and

(iii) Testing the effect of the fractions on H5 gene transcription by quantitative primer extension analysis of RNA isolated from injected oocytes.

3-2 Isolation of chromatin salt-wash fractions

The cells used as the source of the chromatin salt-wash fractions (CSWFs) were the AEV cells described in chapter 2. These cells are chicken erythroid cells which express H5 mRNA and protein, and large numbers can be grown easily in suspension culture. A non-producer cell line (i.e. unable to produce the avian erythroblastosis virus; 9-3-15) was used, to minimise 'contamination' of extracts with viral components.

Initially, approximately 10^9 AEV cells were grown in culture and CSWFs were isolated by the method described in 9-3-16. Increasing concentrations of salt were used to isolate five separate fractions: 150mM, 300mM, 450mM, 600mM and 2M, thereby attempting to achieve an initial purification. [Note that Stunnenberg and Birnstiel (1982) found that sea urchin embryo CSWFs isolated with 450mM, 600mM and 2M salt were inhibitory to sea urchin histone gene transcription in frog oocytes.]

The CSWFs were exhaustively dialysed against bi-distilled water, concentrated by vacuum dessication, resuspended in 'protein injection buffer' (PIB; 9-3-14(v)(d); 9-3-16(iii)), and then stored in small aliquots, at -80 ^{o}C. Approximate protein concentrations were estimated by spectrophotometer readings at 280 nm. Each fraction was found to contain only approximately 200 ug of protein.

Given that the haploid DNA content of a chicken cell is 1.26 pg (Old and Woodland, 1984), then 10^{9} diploid cells should contain approximately 2.5 mg of DNA. Harlow (1974) determined the ratios of total protein, histones and 'non-histone proteins' to DNA in crude chicken erythroblast chromatin. Using these ratios and the figure already noted for total DNA, it can be calculated that approximately 9 mg of protein, comprising about 3.5 mg of histones and about 6.5 mg of non-histone protein, should be obtained from the AEV cell chromatin. [Note that, with regard to the CSWFs, most of the H1 and H5 molecules are likely to be isolated in the 600mM wash, and most of the core histones should be extracted in the 2M wash.]

Therefore, the amount of protein obtained in the CSWF isolation procedure was much lower than would be expected, even allowing for the fact that histones are not readily detected at 280 nm, due to their low content of aromatic amino acids. This suggests that the AEV cell CSWF extraction was inefficient at one or more steps, and/or that losses occurred at certain stages of the procedure. In this regard, it should also be noted that each salt wash was only carried out for 10-15 minutes, in a small volume of buffer.

Although a relatively low yield of protein was obtained in the CSWF extraction, this was not critical to the aims of the project. More important was the fact that enough material was isolated for the oocyte

experiments (which are described in the next two sections). An accurate analysis of the CSWFs was also considered unimportant with respect to the initial aims of this work. CSWF samples were run out on SDS polyacrylamide gels and, as was expected, ladders of bands were obtained. However, no further characterisation of the proteins was carried out.

3-3 Effect of the CSWFs on H5 transcript levels in co-injected oocytes

To test the CSWFs for possible effects on H5 gene transcription, the following experiment was carried out:

(i) pH52.6 DNA was mixed with plasmid DNA containing two chicken histone genes, an H1 and an H2B gene, which acted as internal controls. [This plasmid will be referred to as pH1/H2B in this thesis, but was originally named p7AT-H2A (p7AT minus H2A). It was derived from a subclone of a 7 kb fragment of chicken DNA, containing an H1, an H2A and an H2B gene, in pAT153 (this parent construct is called p7AT) (Sturm, 1985; Sturm et al., 1986; 7-2; figure 7.1; 9-2-4). It should also be noted that the H2B gene in pH1/H2B (and in mH5/H2B - see chapter 4) has a truncated promoter, compared to the gene in p7AT, and this results in a reduced level of transcription in frog oocytes (Sturm, 1985; Sturm et al., 1986).]

(ii) A sample of each of the 5 CSWFs was injected into the cytoplasm of separate batches of frog oocytes. About 3 to 4 hours later the mixture of plasmid DNAs was injected into the nucleus of these oocytes (figure 3.1(a)); the 3 to 4 hours should allow time for the nuclear proteins in the CSWFs to migrate to the oocyte nucleus, before the DNA is injected (Stunnenberg and Birnstiel, 1982; Dingwall et al., 1982; De Robertis, 1983; see chapter 7). Approximately 200 ng of protein (in 50 nl) and 5 ng of DNA (in 25 nl) were injected into each oocyte. One batch of oocytes was injected with PIB, instead of a CSWF, as a control.

[It should be noted that generally in the CSWF experiments plasmid

DNAs were mixed together in ratios which ultimately generated reasonably equivalent levels of transcripts for each of the genes used. This was done to simplify the subsequent analysis and allow direct comparisons to be made between the primer extension products on the one autoradiograph.]

(iii) After incubation for approximately 24 hours, total oocyte RNA was isolated and analysis was carried out by primer extension with three specific 26-mers (one for each of the histone genes injected; 9-3-12). For simplicity, and to eliminate possible artifacts, all three primers were used in the same reaction. Each primer was used in excess (20 - 30 fold) over the corresponding RNA, to allow quantitative analysis. The result is shown in figure 3.1(b).

For each of the histone transcripts two or more major bands are present, representing the use of more than one cap site and/or premature termination (or other artifacts) of reverse transcription (as noted in section 2-2(1)). The sizes of the major bands obtained were as expected:

H5 : 111 bases (section 2-2(1))

H2B : 68 bases (Sturm, 1985; Sturm et al., 1986; Younghusband et al., 1986)

H1 : 58 bases (Sturm, 1985; Sturm et al., 1986; Younghusband et al., 1986).

It can be seen that there is some variability in the overall intensity of the bands generated by the primer extension products, between the different tracks. This could be due to the effect of the different CSWFs but the results of other experiments suggest that it is actually due to variability in the injection or RNA isolation techniques, or to the differential survival of oocytes between individual batches.

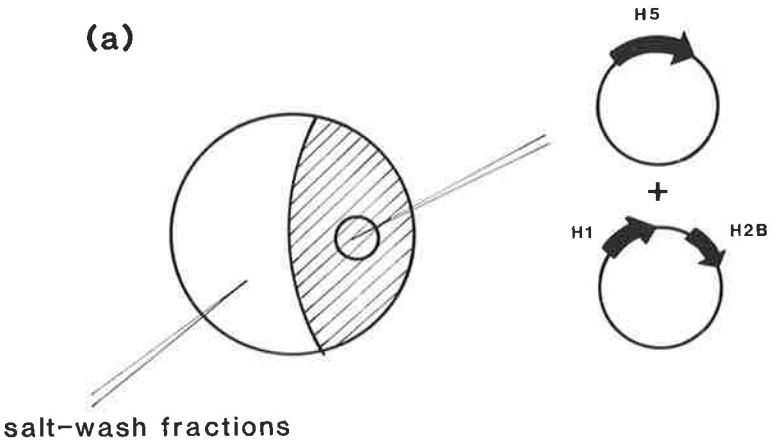
More importantly, figure 3.1(b) shows that when the CSWFs are

Figure 3.1 : Effect of the AEV cell CSWFs on the level of H5 transcripts in co-injected oocytes

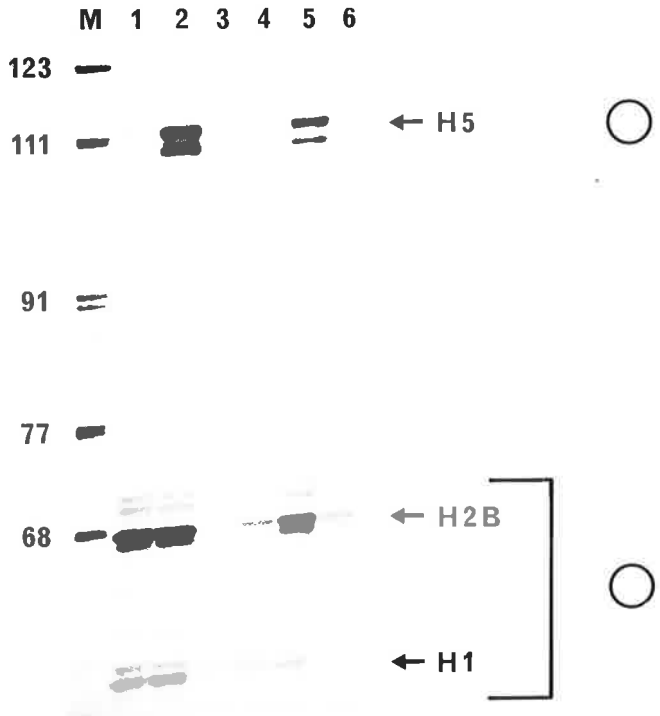
(a) This diagram is a representation of an experiment in which pH52.6 and pH1/H2B DNA were co-injected, with the AEV cell CSWFs, into frog oocytes. The CSWFs were injected into the cytoplasm of the oocytes, followed 3 to 4 hours later by nuclear injection of the DNA mixture.

(b) The result of primer extension analysis of the RNA isolated from the co-injected oocytes is shown. Track M is a marker track, in which end-labelled HpaII-cut pBR322 DNA was run; sizes are shown in bp. Track 1 represents the injection of protein injection buffer (PIB), instead of a CSWF, as a control. Tracks 2 to 6 represent the injection of the five CSWFs: 2M, 600 mM, 450 mM, 300 mM and 150 mM, respectively. The H5, H2B and H1 extension products are indicated. The circles emphasise that the H1 and H2B genes were linked together on a separate plasmid from the H5 gene.

(a)


H5
+
H1
H2B
salt-wash fractions

(b)


M 1 2 3 4 5 6
123
111
91
77
68
H5
H2B
H1

injected, rather than the PIB, there is a relative increase in the level of H5 transcripts, compared to the H1 and H2B transcripts. This is best seen by comparing the PIB track (1) with the 2M CSWF track (2). [In the 600mM track (3) the bands are barely visible. However, upon close inspection of the autoradiograph it appears that this CSWF does produce the relative increase in the H5 transcript levels, and this has also been confirmed in other injection series; for example, see figure 4.3(b).]

Experiments were then carried out to test that the CSWF effect was repeatable and could be obtained under a range of different conditions. These experiments are outlined below. For simplicity of presentation, figure 3.2 shows the result obtained when a number of different conditions were combined in the one experiment.

3-4 Examination of the CSWF effect

(1) The CSWF effect on the relative level of H5 transcripts was obtained with a further five pH52.6 + pH1/H2B + CSWFs experiments.

(2) Subclones of the H5 gene in M13 vectors were used in place of pH52.6 in co-injection experiments, and the CSWF effect was still observed (see figures 3.2, 4.3, 4.4, 5.6, 6.1 and 6.2)

(3) The relative increase of H5 transcripts was also obtained when a chicken H2A gene was used as a gene control in CSWF co-injection experiments (see figures 3.2, 4.3 and 4.4). [The H2A gene used is part of a plasmid referred to as pH1/H2A in this thesis. This also contains the H1 gene previously mentioned, and again the plasmid is derived from p7AT (see 3-3; Sturm, 1985; Sturm et al., 1986).]

(4) The CSWF effect was also obtained when protein was used as a control rather than "straight" PIB. The protein controls used were: a solution of bovine serum albumin (BSA) in PIB, a mixture of histone proteins from chicken erythrocytes in PIB, and a mixture of histones from

CV-1 cells (monkey kidney cells, grown in culture) in PIB. For cytoplasmic injection approximately 200 ng of protein was introduced into each oocyte. In the nuclear injection procedure (see (5) below) approximately 25 ng of protein was introduced. None of the protein solutions produced the CSWF effect on the relative levels of H5 transcripts. Results obtained using BSA as a control are shown in figures 4.3, 5.6 and 6.1.

(5) The CSWF effect was still observed when DNA was mixed with a CSWF and a single injection into the oocyte nucleus was performed. Originally the double injection procedure was used because of the experience of Stunnenberg and Birnstiel (1982) that this eliminated the problem caused by DNases contaminating their CSWFs. However no such problem was encountered with the AEV cell CWSFs. Therefore, because the nuclear co-injection method was much easier and faster to perform than the double injection procedure, the former was used in all subsequent experiments.

In the nuclear co-injection protocol, the DNA and the CSWF were mixed together, pre-incubated at room temperature for about 15-20 minutes, and this mixture was kept on ice, or stored frozen at -20^{o} C overnight, before injection. A ratio of approximately 5 ng of DNA to 25 ng of protein was usually used in the DNA + CSWF mixtures.

(6) CSWFs were isolated a further two times, from two individual batches of approximately 10^{9} AEV cells, using essentially the same procedure utilised in the first isolation (CSWF I). For the first new lot of CSWFs (CSWFs II), five salt extractions were again carried out. Following dialysis and concentration, approximate protein concentrations were estimated by spectrophotometry; each fraction was found to contain about 300 ug of protein. CSWF III was isolated with a single (one hour) 2M salt wash only, to minimise handling. [Unlike the case with the experiments

of Stunnenberg and Birnstiel (1982), no general inhibition of transcription was observed with CSWFs isolated with the higher salt concentrations; for example, see figure 3.1.] Approximately 1.3 mg of protein was contained in the dialysed and concentrated CSWF III preparation. As was the case with CSWF I, the amount of protein isolated in CSWFs II and III was relatively low, but was nevertheless quite sufficient for experimentation.

All five of the CSWF II fractions were found to produce a relative increase in the level of H5 transcripts in co-injected oocytes. Results obtained using a CSWF II fraction are shown in figures 3.2, 5.6(b) and 6.1. CSWF III was also found to produce the same effect in the oocyte co-injection experiment.

Overall, the CSWF effect was obtained in 20 independent sets of experiments, each involving approximately 10 to 20 batches of injected oocytes, over a period of about 2 years. Three individual batches of AEV cell CSWFs (and a fourth nuclear fraction, of a different type - see chapter 6) were isolated which produced the relative increase in H5 transcript levels in co-injected oocytes.

As mentioned above (3-3), figure 3.2 shows the result obtained when a number of the conditions discussed in (1) - (6) were adopted in the one experiment. Specifically, this involved:

- the H5 gene in an M13 subclone
- pH1/H2A as the gene control plasmid
- a sample from the second batch of CSWFs
- the nuclear co-injection method.

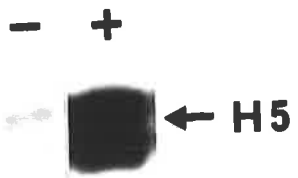
Other examples of the CSWF effect are presented in the next 3 chapters, in figures 4.3, 4.4, 5.6, 6.1 and 6.2.

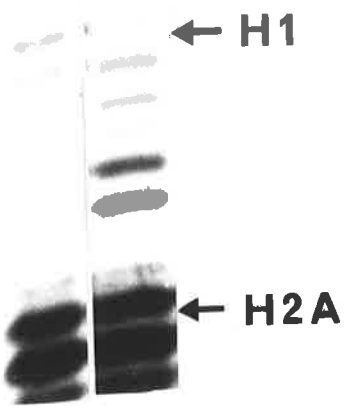
Figure 3.2 : Obtaining the CSWF effect under different experimental conditions

A mixture of mH5/-174 DNA (see chapter 5), pH1/H2A DNA and a sample of the second preparation of CSWFs was injected into the nucleus of each of a batch of oocytes. PIB was injected, with the DNA mixture, into a separate batch, as a control.

The [-] track represents primer extension analysis of RNA from the PIB-injected oocytes. The [+] track represents extension on RNA from the CSWF-injected oocytes. The H5, H1 and H2A extension products are indicated. The major H2A extension products are 48 and 49 bases in length (Sturm, 1985; Sturm et al., 1986). Note that the prominent bands between the H1 and H2A extension products represent premature termination of reverse transcription on H5 transcripts.

[It should also be noted that, as discussed for the H2B gene (3-3), the H2A gene in pH1/H2A has a truncated promoter, compared to the gene in p7AT (see 3-3 and chapter 7), which results in a reduced level of transcription in frog oocytes.]


−
+
H5


H1
H2A

One aspect which has not yet been discussed is the question of repeatability with oocytes from different frogs. With regard to this, two conclusions can be drawn from the co-injection experiments that were carried out. Firstly, the CSWF effect on the relative level of H5 transcripts was obtained with oocytes from different frogs. Secondly, however, with the oocytes from some frogs the effect was not observed at all.

In total, the CSWF effect was obtained with the oocytes from 11 frogs, while no (or an ambiguously small) effect was obtained with the oocytes from 7 frogs. No correlations could be observed between the physical properties of the frogs or the oocytes and the capacity to obtain the CSWF effect. In this regard, however, there did appear to be some correlation between high general expression levels from all of the injected templates and the production of the CSWF effect, but this was not observed in all cases.

Variability in the responses of oocytes from different frogs has been noted by other workers (e.g. Korn and Gurdon, 1981; Jones et al., 1983; Anna Koltunow, this department, personal communication; Jason Loveridge, this department, personal communication). In the case of the CSWF effect, the variability suggests that an oocyte component may be involved in the effect and this factor varies in its amount or properties between oocytes from different frogs (see chapter 8).

It was also observed that samples of the CSWFs which were being used in experiments did not continue to give the CSWF effect indefinitely. Although fractions which had been stored frozen at -80 °C for over a year were still able to produce the CSWF effect, fractions which were thawed and re-frozen 3 or 4 times often lost the capacity to produce the effect. This may simply indicate that thawing and re-freezing causes breakdown of an

active component of the CSWFs (see chapter 8). This loss of CSWF activity, and the variability in response exhibited by the oocytes from different frogs, were two major difficulties encountered with the CSWF co-injection experiments.

3-5 Discussion

The results of the experiments described above show that CSWFs isolated from the AEV-transformed chicken erythroid cells give rise to a relative increase in the level of H5 transcripts when co-injected, with the H5 gene, into Xenopus oocytes.

This effect cannot be due to the introduction of H5 mRNA from the AEV cells into injected oocytes, which is subsequently isolated and detected in primer extension assays. This conclusion can be drawn due to the fact that the AEV cell H5 mRNA generates an extension product which is 9 bases longer than that generated by H5 transcripts from injected oocytes (refer to chapter 2).

It is also highly unlikely that the CSWF effect is caused by ionic components of the fractions, or other small molecules, since the CSWFs were exhaustively dialysed against bi-distilled water. Also, the CSWFs were injected into oocytes in PIB, which was used as a control in the co-injection experiments.

There are two possible ways of interpreting the CSWF effect: the relative increase in the level of H5 transcripts could be due to a specific effect on the H5 gene or transcript, or could be the result of a decrease in the level of the H1, H2A and H2B transcripts.

The former interpretation is favoured by the observation that, in general, oocytes injected with CSWFs survived less well than those injected with PIB alone. This often led to a generally lower level of intensity of

the resultant primer extension products. Therefore, the bands in the 'CSWF tracks' were either of the same, or a lower, intensity compared with the 'PIB' tracks (e.g. see figure 3.1(b)). Given this, results of the type presented in figures 3.1(b) and 3.2 suggest that the CSWFs give rise to an increase in the level of H5 transcripts in co-injected oocytes.

This interpretation is also supported by evidence presented in the next chapter.

The CSWF effect on H5 transcript levels could be mediated by an increase in the transcription of the H5 gene or an increase in the stability of the H5 transcript (or both). Again, the experiments discussed in the next chapter suggest that the increase is at the level of H5 gene transcription.

It was observed that all five of the CSWFs in the first and second preparations produced an increase in H5 transcript levels when co-injected into oocytes with the H5 gene. There are a number of possible explanations for this result. Firstly, the CSWF isolation procedure may have been inefficient at each salt-wash step, resulting in the isolation of many of the same factors in each CSWF. Secondly, there may be more than one factor capable of producing the CSWF effect. Thirdly, if only one factor is responsible for the effect it may occur in the nucleus in more than one form; for example, one form loosely associated with the chromatin and another tightly bound to the DNA.

Finally, it should be noted that the CSWFs were isolated from a retrovirus-transformed cell line and not from cells taken directly from chicken blood or bone marrow. The AEV cells offered many advantages, including the ease with which large numbers of them could be grown and the fact that they represented a relatively pure population of chicken erythroid cells, blocked at an early stage of differentiation, which were

expressing H5 mRNA and protein. In the non-producer AEV cell line used only two AEV proteins are synthesised, p75(gag-erb A) and gp65(erb B), and neither of these are located in the cell nucleus; p75 is found in the cytoplasm and gp65 is located in the plasma membrane (Graf and Beug, 1983). Thus, it appears highly unlikely that a viral protein is responsible for the CSWF effect on H5 transcript levels.

Therefore, the experiments discussed in this chapter suggest that one or more factors which are normally present in chicken pre-erythroblasts can produce an increase in H5 transcript levels when co-injected, with the H5 gene, into Xenopus oocytes. Furthermore, this effect is specific for the H5 gene, compared with the chicken H1, H2A and H2B genes used in the experiments. [These genes provide relevant controls since, like the H5 gene, they are expressed in the AEV cells. Also, as chicken histone genes, they are closely related to the H5 gene. In particular, sequence comparisons suggest that the H1 and H5 genes were derived from a common ancestral gene (Coles and Wells, 1985).]

CHAPTER 4

NATURE OF THE SALT-WASH FRACTION EFFECT: TRANS-STIMULATION OF H5 GENE TRANSCRIPTION

CHAPTER 4 - NATURE OF THE SALT-WASH FRACTION EFFECT: TRANS-STIMULATION OF H5 GENE TRANSCRIPTION

4-1 Introduction

This chapter describes experiments designed to show at which level the CSWFs were exerting their effect: at the level of H5 gene transcription or H5 mRNA stability. The experiments which provided a resolution of this problem involved the use of a construct which contained both the H5 gene and the chicken H2B gene used as a control in some of the co-injection experiments described in the previous chapter. This construct was originally made to provide a more convenient way to co-inject the H5 gene with a control gene. However, co-injection of this construct with the CSWFs yielded an unexpected result which suggested that the CSWFs act to increase H5 gene transcription.

The construction of the H5-H2B clone and the results obtained when this construct was used in CSWF co-injection studies are described here, together with an experiment carried out as a follow up to these studies, which provides further evidence that the CSWFs do act directly on the H5 gene and not on the H1, H2A or H2B genes (refer to section 3-5).

4-2 Construction of mH5/H2B

The source of the H5 gene used in this cloning procedure was the plasmid pH52.6, described in chapter 2. The H5 gene could be excised from the plasmid with a HindIII / BamHI digestion. The H2B gene used came from p7AT (see chapter 3), and a 1.85kb XhoI-XhoI fragment, containing the gene, had been previously isolated by Dr. R. Sturm.

A 2.6kb fragment, containing the H5 gene, was excised from pH52.6 using a HindIII / BamHI double digestion and the fragment was isolated by extraction from low melting point agarose (9-3-4(iii), 9-3-7). The 1.85kb XhoI-XhoI fragment containing the H2B gene was cloned into a SalI M13mp8

vector (9-3-8, 9-3-10) and dideoxy sequencing (9-3-11) was performed to confirm the isolation of recombinants. A clone (mH2B.XX) which carried the H2B gene in the orientation shown in figure 4.1(a) was identified and the replicative form (RF) of the phage DNA was prepared (9-3-2). The H2B gene was then isolated from this DNA as a 1.85kb HindIII-BamHI fragment.

The HindIII-BamHI H5 and H2B fragments were ligated together and a sample of the ligation mixture was run on a vertical, 1% low melting point agarose gel (9-3-4(ii)), next to a marker track of lambda phage DNA digested with HindIII. A 4.45kb band, representing a single H5 gene (2.6kb) joined to a single H2B gene (1.85kb), was cut out of the gel and the DNA was isolated.

Two types of fragment would have been present in this DNA: a fragment with BamHI ends and a fragment with HindIII ends. In order to avoid possible transcriptional interference in the subsequent injection experiments, it was desirable to use the H5-H2B fragment with the BamHI ends, since in this fragment the direction of transcription of the genes is divergent (see figure 4.1(b)). Therefore, the 4.45kb DNA mixture was put in a ligation reaction with a BamHI M13mp83 vector, to select exclusively for the BamHI-BamHI fragment.

The isolation of recombinants was confirmed by sequence analysis and a clone which gave H5 3' end sequence was selected for further characterisation. Firstly, the presence of both genes was demonstrated by dot blot analysis, using nick translated H5 and H2B gene probes (9-3-13). Secondly, diagnostic restriction enzyme digestions were performed on RF DNA, as shown in figure 4.2(a) (and refer to figure 4.1(b) for the location of restriction sites). BamHI digestion (track B) generated a vector band and a band at a position expected for a 4.45kb H5-H2B fragment. A BamHI /

Figure 4.1 : Construction of mH5/H2B

(a) mH2B.XX, used in the construction of mH5/H2B and in the experiment described in section 4-4, is schematically represented here. The orientation of the H2B gene is shown relative to the unique sites in the M13mp8 polylinker.

(b) mH5/H2B is represented together with relevant restriction sites and fragment sizes.

B = BamHI

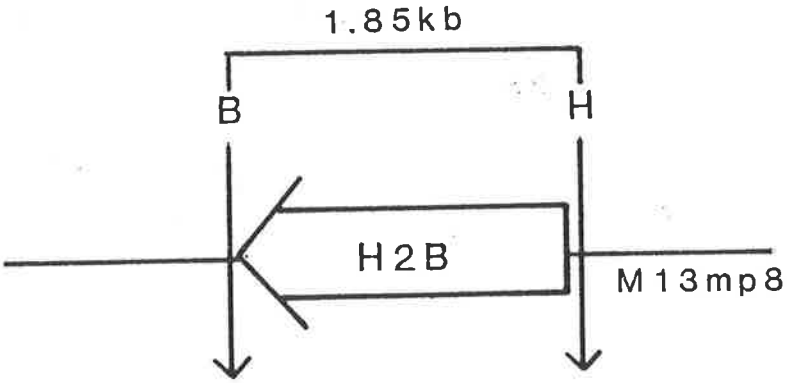
H = HindIII

S = SacII

kb = kilo-base pairs

b = base pairs

(a) mH2B.XX



(b) mH5/H2B

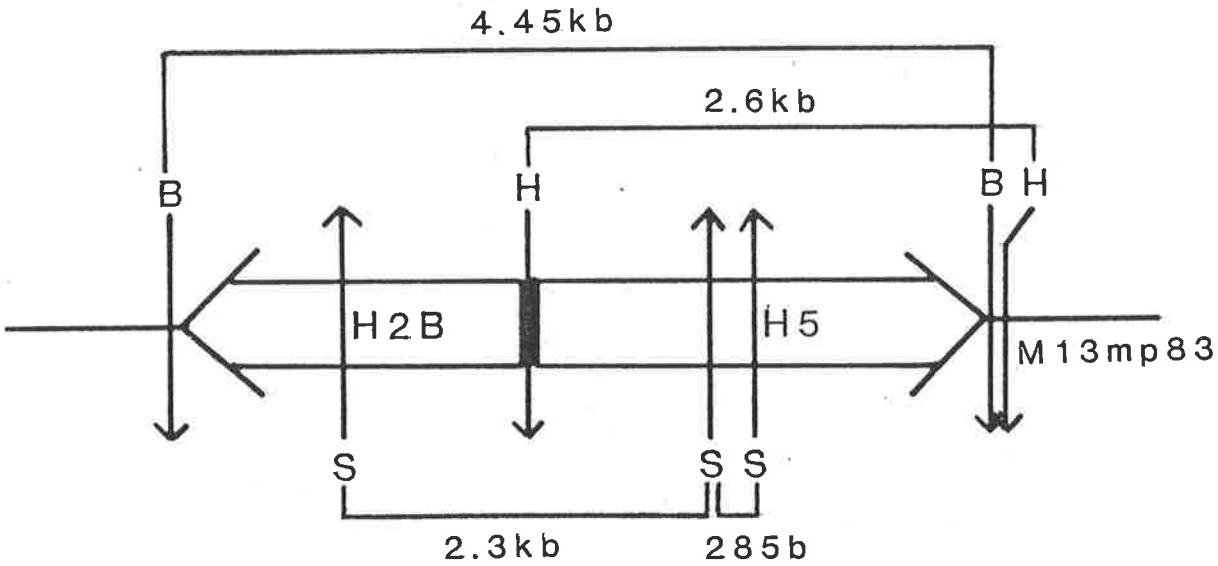


Figure 4.2 : Restriction enzyme analysis of mH5/H2B and mH2B.XX

(a) The result of three digestions of mH5/H2B DNA is shown. Electrophoresis was carried out using a horizontal 1% agarose gel.

B = BamHI

B/H = BamHI/HindIII (double digestion)

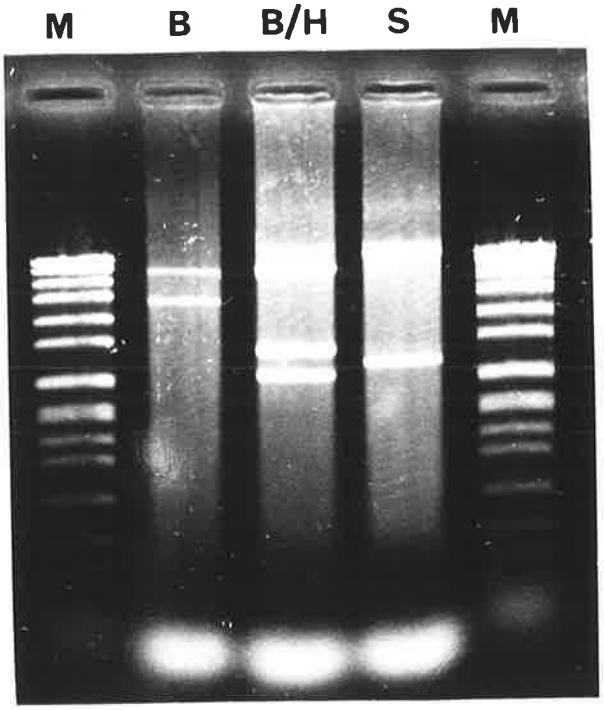
S = SacII

The two marker tracks (M) represent bacteriophage SPP-1 DNA digested with EcoRI (obtained from BRESA). The sizes of the marker fragments in kilo-base pairs (kb) are 7.84, 6.96, 5.86, 4.69, 3.37, 2.68, 1.89, 1.80, 1.45, 1.33, 1.09, 0.88, 0.66, 0.48 and 0.38.

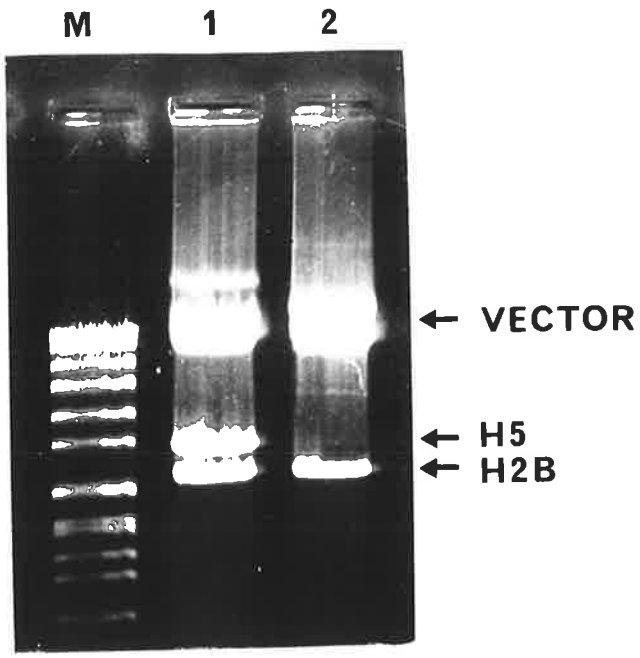
The relevant restriction sites in mH5/H2B are indicated in figure 4.1, together with the sizes of the fragments excised by the enzymes. BamHI digestion removes the 4.45 kb H5-H2B insert from the 7 kb M13 vector. BamHI/HindIII digestion excises the 2.6 kb H5 gene fragment and the 1.85 kb H2B gene fragment. Digestion with SacII removes a 2.3 kb fragment (and a 285 bp fragment which is not visible).

(b) The result of an EcoRI/HindII double digestion of mH5/H2B DNA is shown in track 1. The EcoRI site of M13mp8 (or 83) is located next to the BamHI site. Therefore, EcoRI/HindIII digestion yields the same pattern as BamHI/HindIII digestion; the H5 and H2B gene fragments are excised from the vector. Track 2 shows that EcoRI/HindIII digestion of mH2B.XX DNA yields only the 1.85 kb H2B gene fragment.

(a)



(b)



HindIII double digestion (track B/H) generated a vector band and bands at 2.6kb and 1.85kb, corresponding to the HindIII-BamHI H5 and H2B fragments, respectively. SacII digestion (track S) yielded the 2.3kb fragment expected from the location of SacII sites within each gene; there are no SacII sites in M13mp83.

Finally, injection of RF DNA into frog oocytes resulted in the production of both H5 and H2B transcripts, as judged by primer extension analysis (see figure 4.3).

The clone characterised by the procedure just outlined was named mH5/H2B and is depicted in figure 4.1(b).

4-3 Co-injection of mH5/H2B with CSWFs

mH5/H2B and pH1/H2A DNA were added together and this mixture was used in a co-injection experiment with each of the five CSWFs from the first preparation (refer to chapter 3). This experiment is diagrammatically represented in figure 4.3(a). Specifically, for each CSWF a sample of the fraction was mixed with the DNA mixture and a single nuclear injection was performed with each of a batch of 25 oocytes. A solution of BSA (in PIB) was used as a control, in place of a CSWF, in one batch. Approximately 5 ng of DNA and 25 ng of protein were injected into each oocyte. The result of primer extension, with the four specific 26-mers, on RNA from the co-injected oocytes is shown in figure 4.3(b).

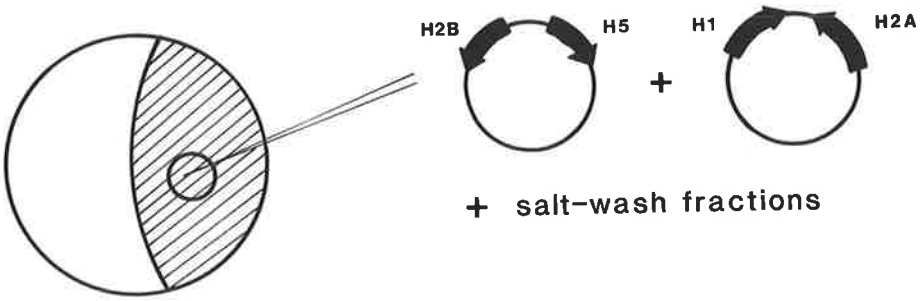
It can be clearly seen that co-injection of the CSWFs (tracks 2 to 6), rather than BSA (track 1), resulted in a relative increase in the level of both the H5 and H2B transcripts. This result was obtained in a total of four sets of experiments, with samples of each of the three preparations of CSWFs (see chapter 3), and with oocytes taken from three different frogs. Also, the oocytes from two other frogs did not give the CSWF effect with the mH5/H2B + pH1/H2A + CSWFs experiment, illustrating again the frog

Figure 4.3 : Enhancement of transcription by the AEV cell CSWFs

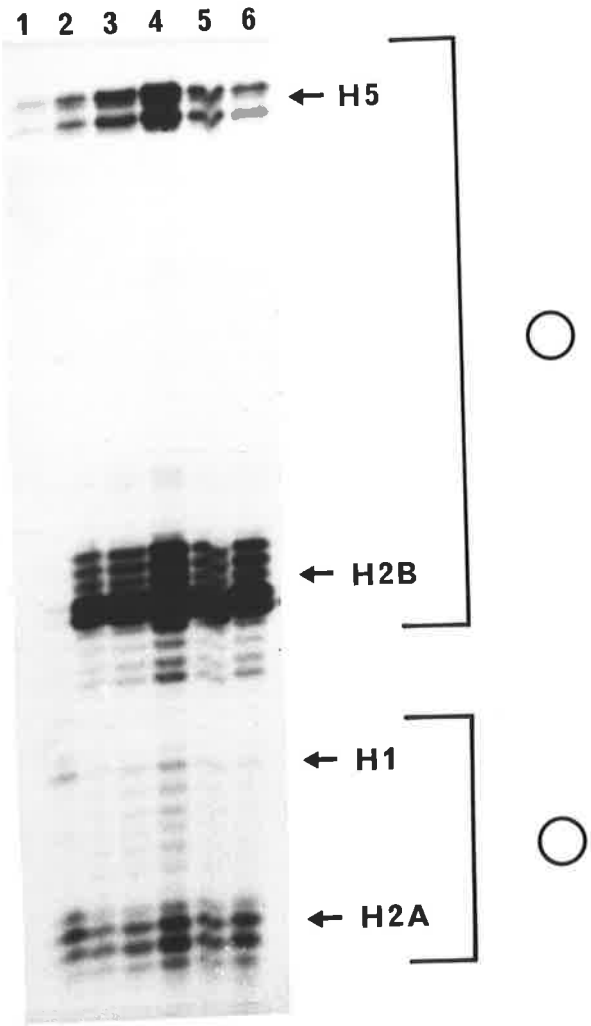
(a) The diagram is a schematic representation of an experiment in which mH5/H2B DNA, pH1/H2A DNA and samples from the first preparation of AEV cell CSWFs were mixed together and injected into oocyte nuclei.

(b) The result of primer extension analysis of RNA from the injected oocytes is shown. Track 1 represents the injection of BSA (in PIB), instead of a CSWF, as a control. Tracks 2 to 6 represent the injection of samples of the five CSWFs: 2 M, 600 mM, 450 mM, 300 mM and 150 mM, respectively. The H5, H2B, H1 and H2A extension products are indicated. The circles emphasise that the H5 and H2B genes were linked on one plasmid, and the H1 and H2A genes were linked on a separate plasmid.

(a)



(b)



variability which is observed with this effect (refer to section 3-4).

As discussed in chapter 3, when the H5 and H2B genes are on separate plasmids, the CSWFs produce a specific increase in the level of H5 transcripts (figure 3.1). However, when the same two genes are present on the one plasmid, the CSWFs produce an increase in the level of both transcripts (figure 4.3). This suggests that :

(1) The CSWFs exert their effect at the transcriptional level, rather than at the level of mRNA stability. This conclusion is based on the reasoning that linking two genes together may affect their transcription but is extremely unlikely to affect the stability of their mRNAs. Ample pecedents exist for such transcriptional effects, e.g. the action of enhancer elements (1-3-4(4)). However, there appear to be no examples of mRNA stability effects resulting from the linkage of two (or more) genes on the one DNA molecule. Therefore, the fact that the CSWFs only produce an increase in the level of H2B transcripts when the H2B gene is linked to the H5 gene strongly suggests that the CSWFs exert their effect on the H5 and H2B genes at the transcriptional level.

(2) A region (or regions) within the H5 gene can act as a transcription enhancer in the presence of the CSWFs. The finding that the H2B gene is only affected by the CSWFs when linked to the H5 gene suggests that at least one region of the H5 gene is involved in the ability of the CSWFs to stimulate transcription. In the mH5/H2B construct, the two genes are divergently transcribed. Therefore, the region of the H5 gene required for the CSWF effect must act in both orientations to affect transcription of both the H5 and H2B genes. Thus, in the presence of the CSWFs this region possesses at least some of the properties of a transcription enhancer element (1-3-4(4)). Also, since the H5 and H2B promoters are

separated by approximately 1kb in mH5/H2B, the H5 gene region required for the CSWF effect must act over a relatively large distance (unless there is more than one region capable of mediating the effect).

The discussion just presented represents the most likely interpretation of the results obtained in the CSWF co-injection experiments. However, there is still one other possible interpretation which must be considered. As argued in chapter 3 (3-5), the evidence is consistent with the conclusion that the CSWFs produce an increase in the level of H5 transcripts in co-injected oocytes, rather than a decrease in the level of H1, H2A and H2B transcripts. The result of the mH5/H2B experiments would also seem to support this view. However, it is still possible, albeit unlikely, that there is a repressor for H1, H2A and H2B transcription in the CSWFs which interacts with a sequence present in the pH1/H2A and pH1/H2B plasmids. Subcloning the H2B gene into M13 (in the construction of mH5/H2B) may remove the gene from direct linkage with this sequence and therefore transcription of the H2B gene would no longer be repressed by the CSWFs. Thus, the enhancement of H5 and H2B transcription in the mH5/H2B experiments would actually be a repression of H1 and H2A transcription, relative to H5 and H2B.

To be consistent with results such as that presented in figure 4.3(b), this interpretation requires that the CSWFs must give rise to a large general increase in the level of transcription and/or mRNA stability in co-injected oocytes. Alternatively, the injection of PIB or BSA must decrease transcription and/or mRNA stability in a general way. Both of these possibilities would appear to be unlikely and are not consistent with the fact that in many instances the general level of intensity of the primer extension products in the 'CSWF tracks' is less than that observed in the

'PIB' or 'BSA' tracks, which correlates with the state of the oocytes after post-injection incubation (3-5).

However, to completely dismiss the 'repressor interpretation', a direct experimental test was required. This is described in the following section.

4-4 Use of the H2B gene in M13 as a control in co-injection experiments

The experiment carried out to discriminate between the two interpretations discussed above involved the use of an M13 subclone of the H2B gene. As described in 4-2, this subclone was made, during the construction of mH5/H2B, by ligating a 1.85kb XhoI-XhoI H2B gene fragment into a SalI M13mp8 vector (4-2). The resulting subclone (named mH2B.XX) is diagrammatically represented in figure 4.1(a). Figure 4.2(b) presents restriction analysis which confirms that only the H2B gene is present in mH2B.XX.

RF DNA of an M13 subclone of the H5 gene (mH5/-174, refer to chapter 5), mH2B.XX RF DNA and pH1/H2A DNA were mixed together and used in a co-injection experiment. The fraction and oocytes used in this experiment had previously been shown to generate the 'CSWF effect'.

If the 'repressor interpretation' described in 4-3 was correct, then a similar result to that obtained for the mH5/H2B + pH1/H2A + CSWFs experiment should have been obtained in this experiment; i.e. since the H2B gene had been removed from the repressor-associated sequence, it should have shown the same 'response' to the injection of CSWFs as the H5 gene - an 'increase' in transcription relative to the repressed H1 and H2A genes. Alternatively, if the CSWFs generate a trans-stimulation of H5 gene transcription, the level of H5 transcripts produced should be increased relative to H1, H2A and H2B transcripts (when the H2B gene is present in

mH2B.XX and not linked to the H5 gene).

Figure 4.4 presents the result obtained in the mH5/-174 + mH2B.XX + pH1/H2A co-injection experiment. Two exposures of the same 3 primer extension tracks are shown. The (-) track represents the injection of PIB and the (+) tracks represent injection of the fraction previously shown to produce the 'CSWF effect'. It can be seen that injection of the latter gives rise to a relative increase in the level of H5 transcripts, compared to the H1, H2A and H2B transcripts. This is best seen by comparing the (-) track in (b) with the (+) tracks in (a).

Following the discussion presented above, this result argues strongly for the hypothesis that the CSWFs produce a trans-stimulation of H5 gene transcription in co-injected oocytes.

4-5 Discussion

Two major results are presented in this chapter. Firstly, use of the mH5/H2B construct in co-injection experiments shows that when the H5 and H2B genes are linked together, the CSWFs produce a relative increase in the level of both H5 and H2B transcripts. Secondly, use of the isolated H2B gene in an M13 subclone (mH2B.XX) in co-injection experiments provides evidence that the CSWFs exert their effect specifically on the H5 gene (and on the H2B gene when it is linked to the H5 gene).

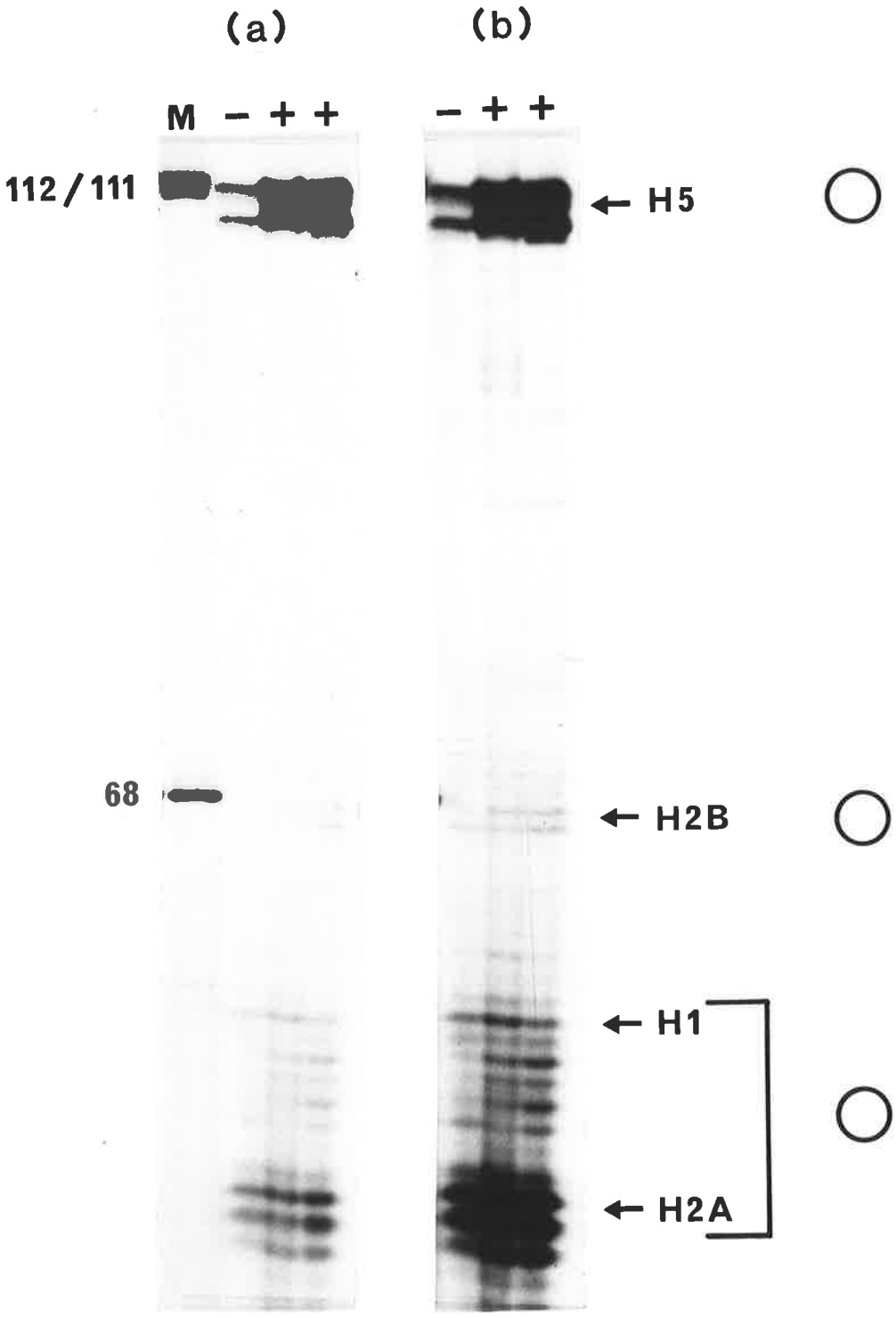
It should be noted that, as seen in figure 4.3(b), the CSWFs appear to stimulate H2B transcription from mH5/H2B to a greater extent than H5 transcription. This was observed a number of times and may indicate that the H2B gene is capable of a higher maximal level of transcription than the H5 gene. This in turn may reflect the abundance and/or activity of oocyte transcription factors which are involved in the expression of these genes.

The results discussed in chapters 3 and 4 indicate that H5 gene sequences, and not vector sequences, are involved in the CSWF trans-

Figure 4.4 : Use of mH2B.XX as a control in a co-injection experiment

An experiment was carried out in which mH5/-174 DNA (see chapter 5), mH2B.XX DNA, pH1/H2A DNA and an AEV cell fraction were mixed together and injected into oocyte nuclei. PIB was injected into a separate batch of oocytes as a control.

Two exposures of the same primer extension result are shown. The marker track (M) shows end-labelled HpaII-cut pUC19 DNA; sizes are in bp. The [-] track represents injection of PIB and the [+] tracks represent injection of the AEV cell fraction. The H5, H2B, H1 and H2A extension products are indicated. The circles emphasise the gene content of the three separate plasmids used in this experiment.


(a)
(b)
M
–
+
+
–
+
+
112 / 111
68
H5
H2B
H1
H2A

stimulation effect. Firstly, the effect is obtained with the H5 gene in either a pBR322 vector or an M13 vector. Secondly, transcription of the H2B gene in an M13 vector is only stimulated by the CSWFs when the H5 gene is also present in the same construct (i.e. compare the results obtained with mH2B.XX and mH5/H2B).

Thus, the results presented in chapters 3 and 4 suggest that the erythroid cell CSWFs can produce a trans-stimulation of gene transcription and this effect involves a region (or regions) of the H5 gene. Furthermore, the trans-stimulation appears to be mediated by an enhancer-like activity.

Once the findings of the experiments described above had been established, two major priorities were considered:

(1) to define a region (or regions) of the H5 gene involved in the CSWF trans-stimulation effect; and

(2) to begin characterisation of the active component(s) of the CSWFs. The experiments which were carried out in these two areas are described in chapters 5 and 6, respectively.

CHAPTER 5

REGIONS OF THE H5 GENE INVOLVED IN EFFICIENCY OF TRANSCRIPTION AND TRANS-STIMULATION BY CSWFs

CHAPTER 5 : REGIONS OF THE H5 GENE INVOLVED IN EFFICIENCY OF TRANSCRIPTION AND TRANS-STIMULATION BY CSWFs

5-1 Introduction

As an initial attempt to define an H5 gene region involved in the CSWF effect, 'gross' deletion mutants of the gene were tested for their ability to exhibit the effect in CSWF co-injection experiments. In addition, these mutants were also utilised to define upstream regions of the H5 gene involved in determining the efficiency of H5 transcription in frog oocytes.

Dr. A. Robins, in this laboratory, had previously constructed a number of Bal31 5' deletion mutants of the H5 gene (in M13). Two of these clones were chosen for use in the oocyte experiments. In addition, two other deletion mutants were constructed using appropriate restriction sites within the H5 gene. This chapter describes the selection / construction of the H5 deletion mutants and their use in defining important regions of the H5 gene involved in transcriptional efficiency and the CSWF effect.

5-2 Selection / construction of 5' deletion mutants of the H5 gene

(1) Selection of Bal31 deletion clones

Two of the Bal31 clones which had already been sequenced were initially chosen as appropriate deletion mutants of the H5 gene. The first of these has its 5'end at -174, with respect to the cap site at +1, and its 3' end at approximately +1360 (as for the H5 gene in pH52.6 - see figure 2.1(a)). The vector used in the construction of this, and the other Bal31 clones, was M13mp9 SmaI/BamHI. The '-174 clone' is here named mH5/-174 and is schematically represented in figure 5.1. The 5' sequence of the H5 gene in this construct was determined by Dr. F. Shannon and subsequently confirmed by me, before use in the oocyte experiments. The location of the 5' end of the gene in mH5/-174, with respect to the H5 gene sequence and

Figure 5.1 : H5 gene deletion clones

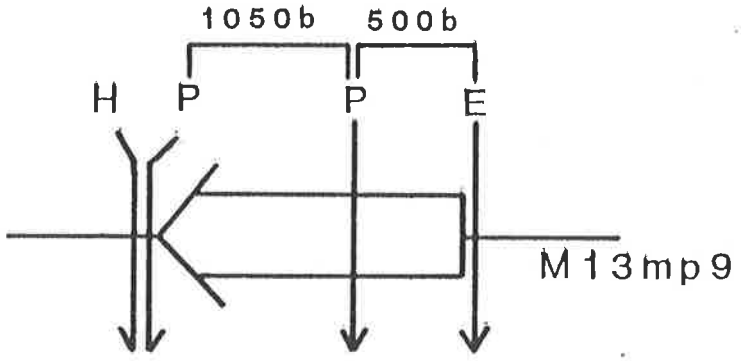
Schematic representations of three of the H5 gene deletion clones are shown, together with relevant restriction enzyme sites. Distances between restriction sites are indicated; b = base pairs.

H = HindIII

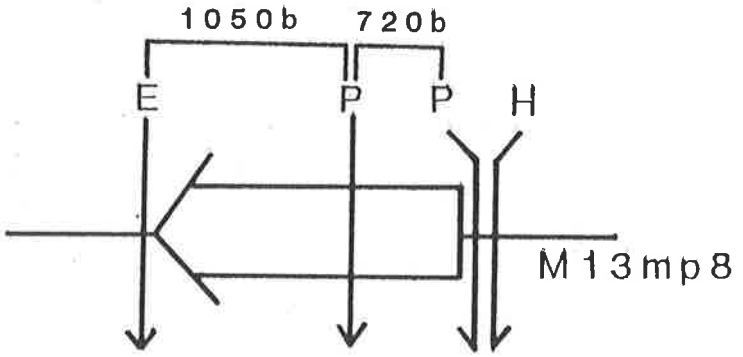
P = PstI

E = EcoRI

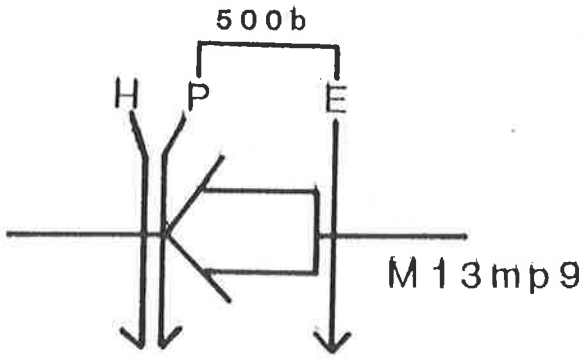
(a) mH5/-174



(b) mH5/-395



(c) mH5/-174(delPP)



potentially important 5' elements, is shown in figure 5.3. It can be seen that -174 is immediately upstream from the region containing the two GC boxes (1-3-4(2); 1-4-4(3)).

The other Bal31 clone used has its 5' end at -85, immediately downstream from the GC box region, as indicated in figure 5.3. This second clone is here named mH5/-85 and is the same as mH5/-174 in all respects, except for the location of the 5'end of the H5 gene. Its 5' sequence was determined by Dr. A. Robins and, again, subsequently confirmed by me.

To obtain another 5' deletion, with its 5' end between -1200 (the approximate 5' end of the H5 gene in pH52.6 - see figure 2.1(a)) and -174, as yet uncharacterised Bal31 clones were screened by sequence analysis (9-3-11). However, no appropriate deletions were identified. Therefore a deletion mutant was constructed, using the convenient SacI site at -395 (see figure 5.3(b)).

(2) Construction of mH5/-395

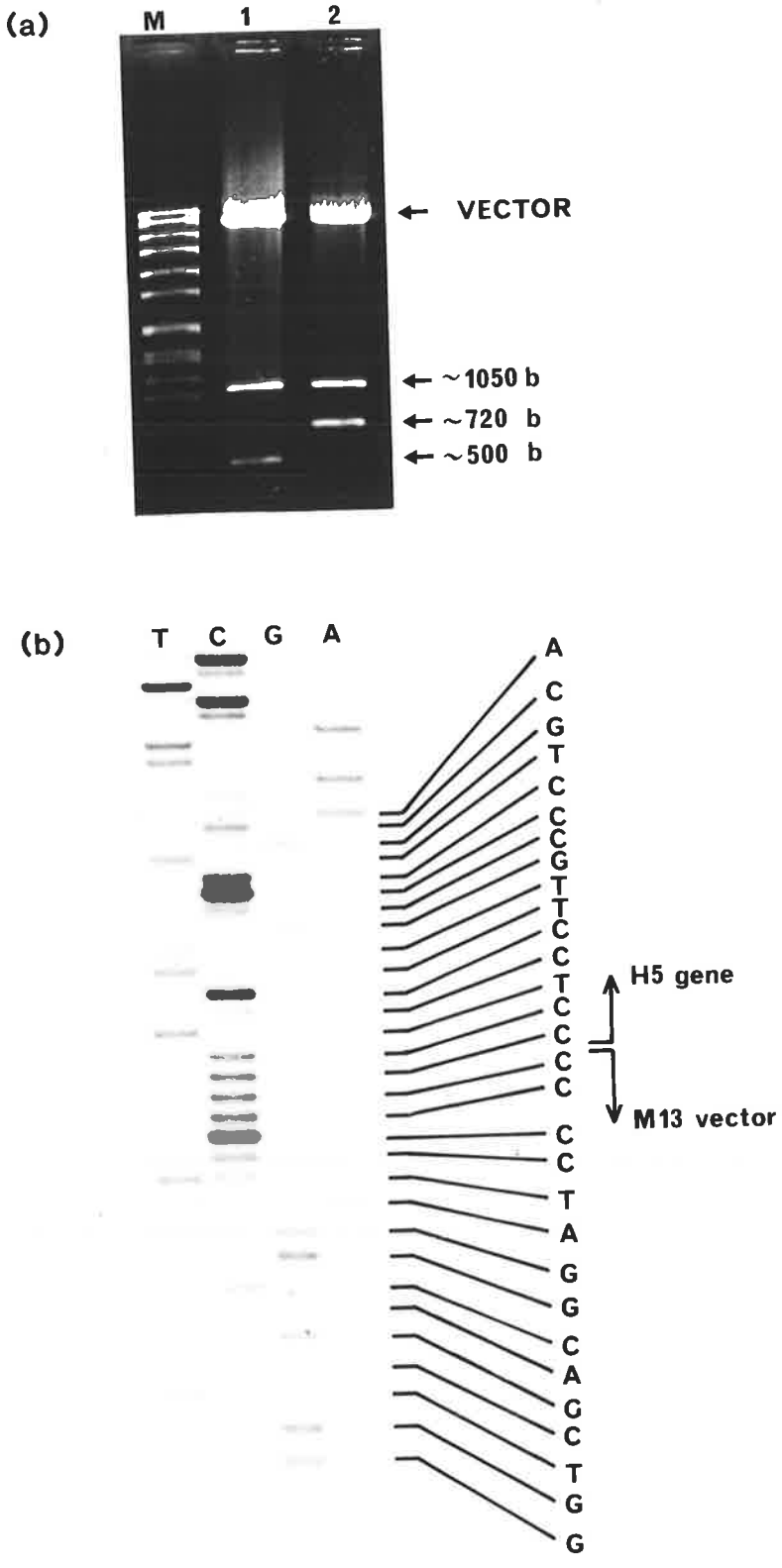
A 1.75 kb SacI - HindIII fragment was excised from pH52.6 and isolated by extraction from low melting point agarose (9-3-4(iii); 9-3-7). This fragment comprised the H5 gene from -395 to (approximately) +1360. The fragment was then blunt-ended and ligated into an M13mp8 SmaI vector (9-3-8(i)). Following transformation (9-3-10), recombinants were screened by sequence analysis (9-3-11), and one of the clones which gave 5' H5 sequence, starting at -395, was selected for further characterisation. The relevant part of the sequence of this clone, named mH5/-395, is shown in figure 5.2(b).

To further analyse mH5/-395, RF DNA of this clone was prepared (9-3-2) and restriction enzyme digestions were performed. An example is shown in figure 5.2(a), and the relevant restriction sites are indicated in figure

Figure 5.2 : Restriction enzyme and sequence analyses of mH5/-395

(a) Restriction enzyme analysis of mH5/-395 RF DNA is shown. The relevant restriction sites are indicated in figure 5.1. The marker track (M) shows electrophoresis of bacteriophage SPP-1 DNA digested with EcoRI; the sizes of the fragments were given in the legend to figure 4.2. For comparison, track 1 represents digestion of mH5/-174 RF DNA with EcoRI/PstI, which excises a 1050 bp fragment and a 500 bp fragment. Track 2 represents digestion of mH5/-395 RF DNA with EcoRI/PstI, which excises a 1050 bp and a 720 bp fragment.

(b) A portion of the sequence of mH5/-395 DNA is presented, to demonstrate the exact starting point of the H5 5' flanking sequence in this clone. The H5 sequence can be compared to that shown in figure 5.3. Sequence analysis was performed using the M13 chain termination method (9-3-11) and priming with the M13 universal sequencing primer.


(a)
M
1
2
VECTOR
~1050 b
~720 b
~500 b
(b)
T
C
G
A
A
C
G
T
C
C
C
G
T
T
C
C
T
C
C
C
C
C
C
C
T
A
G
G
C
A
G
C
T
G
G
H5 gene
M13 vector

5.1(b).

For comparison, track 1 of figure 5.2(a) shows the result obtained when mH5/-174 RF DNA was digested with both EcoRI and PstI (refer to figure 5.1(a) for sites). Three bands are present: a vector band, a band at approximately 1050 bp and a band at about 500 bp. The pattern obtained with the EcoRI / PstI digestion of mH5/-395 (track 2) is the same, except that there is a band at approximately 720 bp instead of 500 bp. This difference can be explained by the extra 221 bp at the 5' end of the H5 gene in mH5/-395 compared with that in mH5/-174.

Thus, overall, the sequence and restriction analyses indicate that mH5/-395 contains the H5 gene from -395 to (approximately) +1360. As already noted, this clone is represented in figure 5.1(b). Also, the location of the 5' end of the H5 gene in mH5/-395, with respect to the 5' flanking sequnce of the gene, is shown in figure 5.3.

5-3 Effect of the 5' deletions on H5 gene transcription in Xenopus oocytes

Constructs of the H5 gene with 4 different 5' ends were now available for use in oocyte experiments. These were: pH52.6, mH5/-395, mH5/-174 and mH5/-85, which contained H5 genes with their 5' ends at -1200, -395, -174 and -85, respectively. These are represented in figure 5.3.

Before their use in CSWF co-injection studies, these constructs were first tested for the level of H5 gene transcription obtained with each of them in frog oocytes. This experiment involved the injection of the same amount of each DNA into separate batches of oocytes, together with a control plasmid, pH1/H2B. Specifically, each H5 clone was mixed with pH1/H2B DNA such that approximately 5 ng of total DNA was injected into each oocyte. As previously described, quantitative analysis was performed by primer extension with the specific 26-mers on total oocyte RNA.

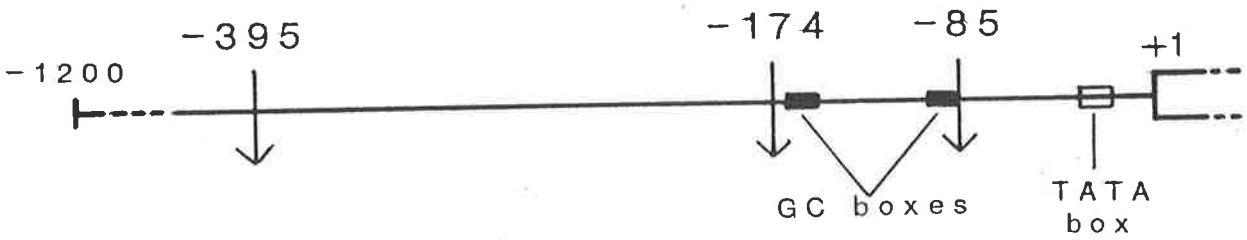
The result of the experiment, showing the H1 extension products as the

Figure 5.3 : 5' deletions of the H5 gene

(a) A schematic representation of the endpoints of the H5 gene 5' deletion constructs is shown. +1 is the start of the transcribed region of the gene. The 'TATA' box and the putative GC boxes of the gene are indicated.

(b) The location of the endpoints of the 5' deletions are shown relative to the 5' flanking sequence of the H5 gene. Numbering is as described for figure 2.1. The SacI restriction site used in the construction of mH5/-395 is indicated. The 'TATA' box and the putative GC boxes are shown spaced away from the rest of the sequence.

(a)


-1200
-395
-174
-85
+1
GC boxes
TATA box

(b)


-395
-400
-350
CAGCACGAGCTCCTCCTTGCCCTGCACATTACCTCCTTGTCTCCTGATCTCCCTCCCAGCACCTATCCTTGTCCT
Sac I
-300
ACCCAGTGTCAGTCCAAAGTACTCCTTTTCCCCATCACATCTCTCCCTGTCCCCTGCTGTCCAACCCAGTGTCCA
-250
-200
CCCTTGTCCCCACCATGTCCATCCCATTCCCACCGTGTCTCACCCTATCCCCACCGTATCCCTCTCCATCCCTTA
-174
-150
ATGTCCAACCCCAGCGT CCGCCC CTTTCCCCATCACATCCCTTCTGGTCCCAACCTCGTCCCTCCCTGCCTGC
-85
-100
-50
CCCAGGCATGTCCTGCG GGGCGG GGCAGAGGGGGGACACGGGGACAGGCAGTCCTCCCCGCGGTCCGTGCCGC
+1
ACCC TTAAAT GCGTGCTGGTGGCGACGCGCGGCCGCAGACGCAGCGGCGGCGGCAGCAGGAGCAGCCCCACAT
+50
+100
CCGTTGTTGCTGGCGGCTCCTTTTTTAAGCTCCCTAACCCCAGTGCCCTGCCGTGGGGTGAAGCGGCGGCC ATG

controls, is presented in figure 5.4. The H2B extension products, although not shown here, gave the same pattern as shown for the H1 products.

Taking the level of intensity of the H1 extension products as equivalent in each track (although see below), the level of intensity of the H5 extension products can be directly compared with each other. This comparison suggests that:

(i) deletion from -1200 (track 1) to -395 (track 2) in the H5 gene does not significantly affect the level of transcription of the gene in frog oocytes;

(ii) deletion from -395 (track 2) to -174 (track 3) produces a large increase in the level of transcription of the H5 gene;

(iii) deletion from -174 (track 3) to -85 (track 4) produces a large decrease in the level of H5 gene transcription (with respect to mH5/-174 transcription).

The experiment described above was carried out three times, using the oocytes from 2 different frogs, and the same result was obtained each time.

An interesting aspect of the results of these experiments is that the transcription from the '-1200 H5 gene' is at almost the same level as that obtained with the '-85 H5 gene'. This suggests that the effects of the stimulator and inhibitor regions essentially cancel each other out in the transcription from the '-1200 gene'. [Actually, it can be seen in figure 5.4 that the level of intensity of the H1 extension product in track 4 is higher than that in the other three tracks. Therefore, when this is allowed for, it appears that transcription from the '-85 H5 gene' is at a somewhat lower level than that from the '-1200 gene'.]

At this stage no further characterisation of the stimulatory and inhibitory regions has been carried out. However, it is possible that the

Figure 5.4 : Transcriptional activity of the H5 gene 5' deletion clones

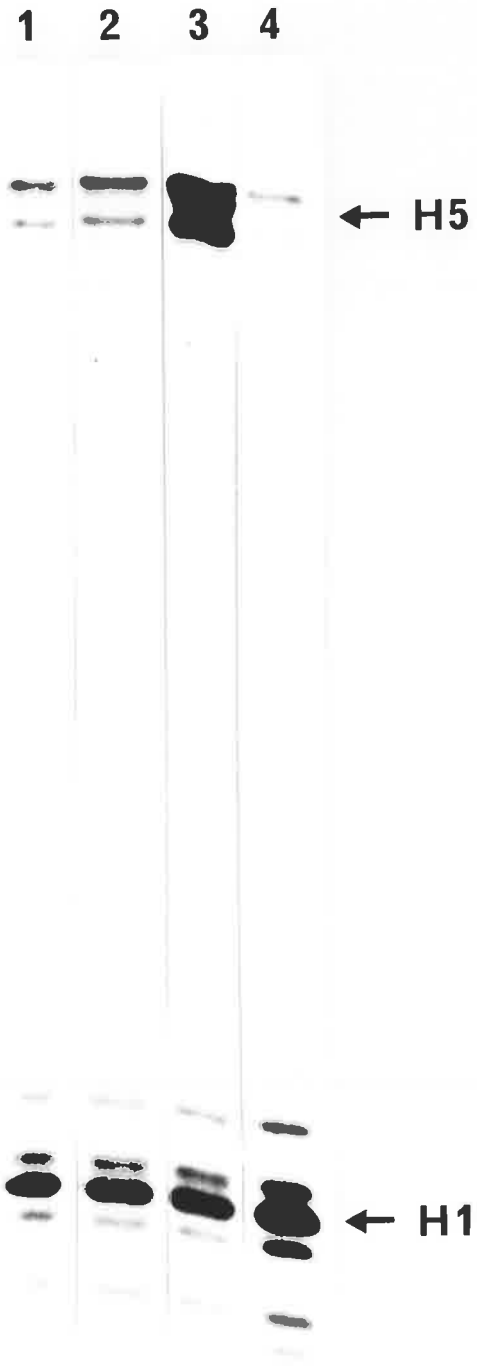
pH52.6, mH5/-395, mH5/-174 and mH5/-85 were independently mixed with pH1/H2B and each DNA mixture was injected into a separate batch of frog oocytes. The result of primer extension analysis of the RNA from these oocytes is shown. The H5 and H1 extension products are indicated. Each track represents injection of a different H5 construct:

Track 1 - pH52.6

Track 2 - mH5/-395

Track 3 - mH5/-174

Track 4 - mH5/-85


1
2
3
4
H5
H1

GC boxes (1-3-4(2); 1-4-4(3)) which are located within the stimulatory region may be at least partly responsible for the activity of this domain (see 5-6 for further discussion).

5-4 Use of 5' deletions of the H5 gene in CSWF co-injection experiments

It has already been shown that the CSWF trans-stimulation effect can be obtained with the '-1200 H5 gene'(chapters 3 and 4). As an initial attempt to define the region involved in this effect, mH5/-174 and mH5/-85 were used in CSWF co-injection experiments.

Firstly, the CSWF effect was obtained with mH5/-174. Furthermore, the effect was observed using samples of both CSWFs I and II, and with oocytes taken from different frogs. The level of stimulation of H5 gene transcription obtained with this construct was the same as that obtained with pH52.6 and mH5/H2B. Results obtained with mH5/-174 are not presented in this chapter, but can be seen in figures 3.2, 4.4, 6.1 and 6.2.

Secondly, full levels of the CSWF effect were also obtained with mH5/-85. Again, this was observed using samples of both CSWFs I and II, and with the oocytes of different frogs. An example of the results obtained with mH5/-85 is presented in figure 5.6(a).

The co-injection studies carried out with the largest of the H5 gene 5' deletions indicate that a region sufficient to generate the CSWF effect is located downstream from -85 (with respect to the cap site). Subsequent to the these experiments, attention was focused on defining a 3' boundary for this region.

5-5 Construction and functional testing of an H5 gene 3' deletion mutant

As an initial attempt to locate a 3' boundary for the H5 gene region involved in the CSWF effect, a large deletion was made of the 3' two-thirds of the H5 gene. Since this deletion removes over half of the transcribed region of the gene, it was thought possible that this could result in a

decreased stability of the H5 transcripts produced in injected oocytes. Therefore, the 3' deletion was made with the H5 gene in mH5/-174, rather than mH5/-85, since the level of transcription from the mH5/-174 H5 gene is much higher than that from the mH5/-85 gene (5-3). It was hoped that this would compensate for any possible decrease in the stability of H5 transcripts made from the 3' deletion clone.

(1) Construction of the H5 gene 3' deletion clone

As shown in figure 5.1(a), there are two PstI sites in mH5/-174, one at +313 in the H5 gene (relative to the cap site) and the other in the polylinker of the vector. The PstI-PstI fragment (approximately 1050 bp) was excised from mH5/-174 RF DNA and the resultant 'vector + remaining H5 gene' fragment was purified through low melting point agarose. The two free PstI ends of this fragment were then ligated together to regenerate circular molecules. Following transformation, 'plaques' were screened by restriction analysis of RF DNA.

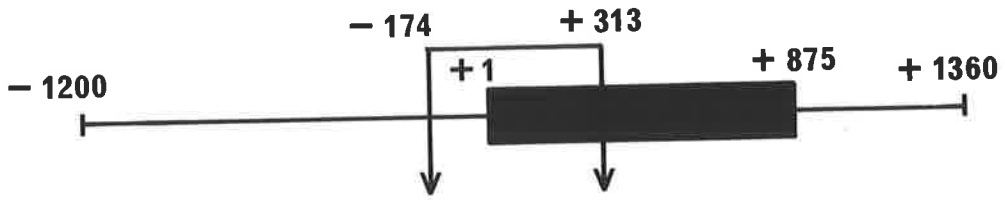
An example of this analysis is presented in figure 5.5(b) and the relevant restriction sites are shown in figure 5.1(a) and (c). Track 1 of figure 5.5(b) shows an EcoRI/HindIII digestion of mH5/-174 RF DNA. The two bands represent the M13 vector, and the H5 gene insert of approximately 1550 bp. Track 2 shows an EcoRI/HindIII digestion of a 3' deletion clone, named mH5/-174(delPP). The insert in this clone is only approximately 500 bp in length, consistent with the insert being the H5 gene from mH5/-174 with the 1050 bp PstI-PstI fragment deleted. Track 3 is an EcoRI/PstI digestion of mH5/-174, showing the H5 gene insert split into the 1050 bp PstI-PstI fragment and the 500 bp EcoRI-PstI fragment. As expected, the same digestion with mH5/-174(delPP) DNA (track 4) gives only the vector band and the 500 bp EcoRI-PstI H5 gene fragment.

Figure 5.5 : The H5 gene deletion construct mH5/-174(delPP)

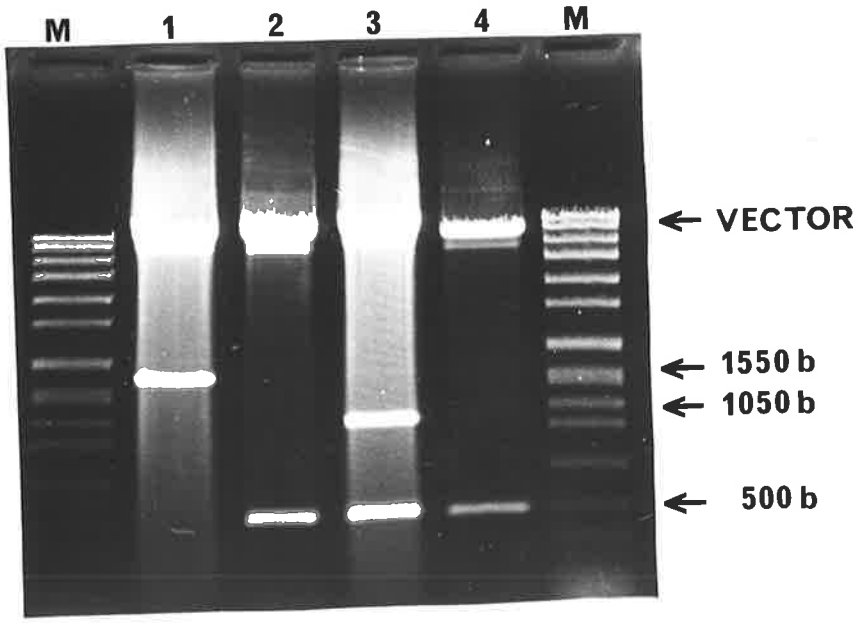
(a) A diagrammatic representation of the H5 gene deletion construct mH5/-174(delPP) is shown. The endpoints of the H5 gene in this construct are -174 and +313, relative to the cap site at +1.

(b) Restriction enzyme digestions of mH5/-174 and mH5/-174(delPP) are shown. The marker tracks (M) show bacteriophage SPP-1 DNA digested with EcoRI; the sizes of the fragments were given in the legend to figure 4.2. Tracks 1 and 3 show mH5/-174 RF DNA digested with EcoRI/HindIII and EcoRI/PstI, respectively. Tracks 2 and 4 show the equivalent digestions of mH5/-174(delPP) RF DNA. The relevant restriction sites are indicated in figure 5.1 and the approximate sizes of the fragments excised from the M13 vectors are shown here (b = base pairs).

(a)



(b)



Thus, restriction analysis indicated that mH5/-174(delPP) was the required 3' deletion clone. This clone is diagrammatically represented in figures 5.1(c) and 5.5(a). The H5 gene in mH5/-174(delPP) has 174 bp of 5' flanking sequence and 313 bp of transcribed region; the 'normal' length of the H5 transcribed region is 875 bp (see figures 2.1 and 5.5(a)).

(2) Use of mH5/-174(delPP) in oocyte experiments

(a) Firstly, mH5/-174(delPP) RF DNA was injected into oocytes to test for H5 gene transcription, and to determine the amount of H5 transcripts obtained, compared with that obtained with mH5/-174. As can be seen in figure 5.6(b), (-) track, H5 transcripts are obtained from oocytes injected with mH5/-174(delPP). Moreover, it was found in other experiments that the level of stable H5 transcripts obtained is equivalent to that obtained from mH5/-174. This indicates that deletion of the 3' two-thirds of the H5 gene transcribed region, including the 3' terminus sites, does not affect the stability of H5 transcripts in frog oocytes. This is consistent with the observation that similar deletions in chicken H2A and H2B genes do not affect the amount of stable transcripts obtained from these genes in injected oocytes (Sturm, 1985; Sturm et al., 1986).

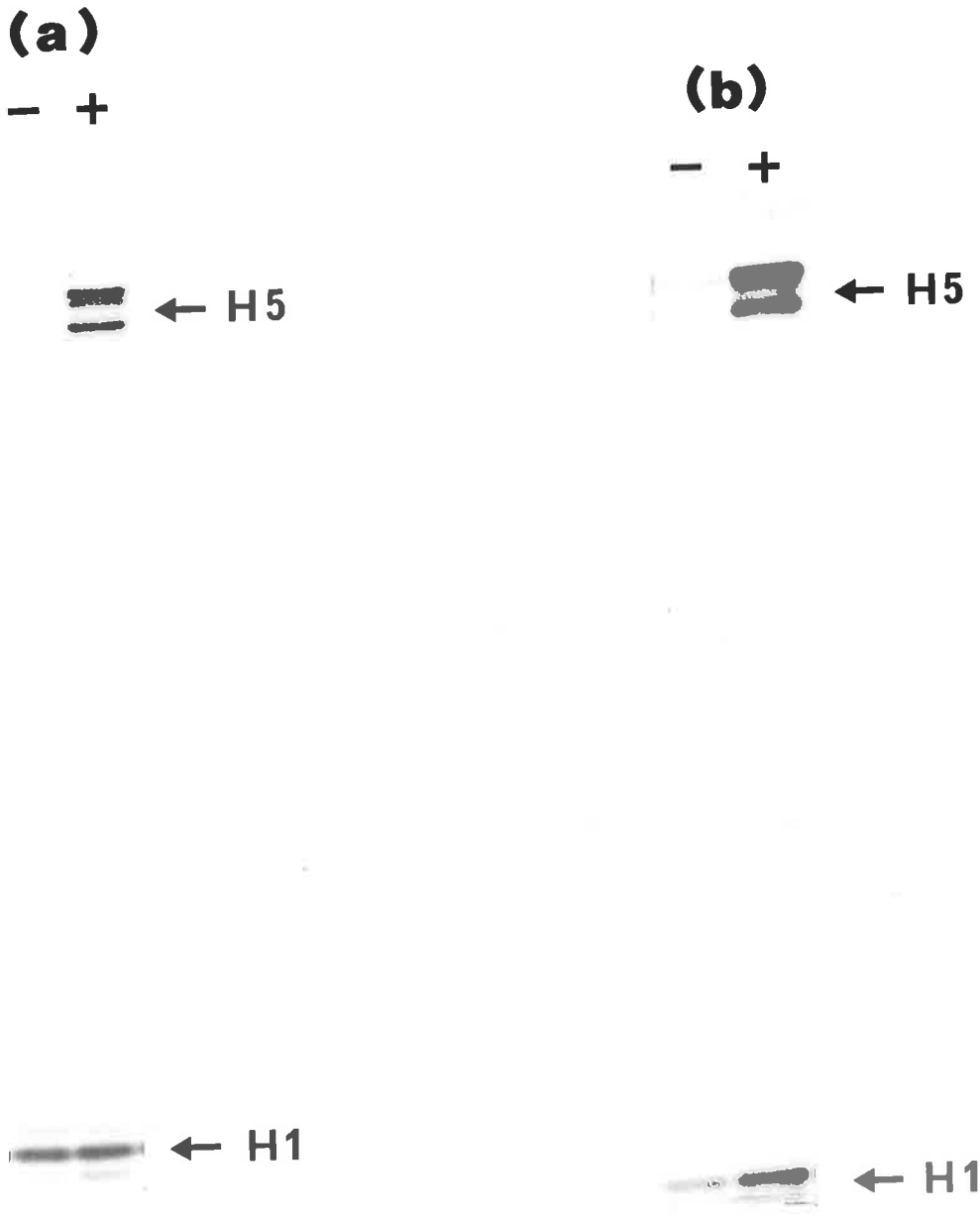
(b) Secondly, mH5/-174(delPP) was tested in co-injection experiments with CSWFs. As shown in figure 5.6(b), full levels of trans-stimulation were obtained with the H5 gene in this clone. This was observed with both CSWFs I and II, and with oocytes taken from two different frogs.

Therefore, a region of the H5 gene upstream from +313 is sufficient to generate the CSWF effect. Furthermore, combining the results obtained with mH5/-85 and mH5/-174(delPP), it appears that the 398 bp region from -85 to +313 in the H5 gene is sufficient to mediate the trans-stimulation of transcription produced by the AEV cell CSWFs.

Figure 5.6 : Result of co-injecting H5 gene deletion constructs with CSWFs

(a) mH5/-85 DNA, pH1/H2B DNA and a sample of the first preparation of CSWFs were mixed together and injected into oocyte nuclei. Bovine serum albumin (in PIB) was injected into a separate batch of oocytes as a control. Primer extension analysis of RNA from the injected oocytes is shown; "+" indicates injection of the CSWF. The H5 and H1 primer extension products are indicated.

(b) A similar experiment was carried out with mH5/-174(delPP) DNA, using pH1/H2A as the control plasmid and a sample of the second preparation of CSWFs. The primer extension result is shown.


(a)
– +
← H5
← H1
(b)
– +
← H5
← H1

5-6 Discussion

(1) Effect of 5' deletions on H5 gene transcription in frog oocytes

The first part of this chapter describes the identification of two upstream regions of the H5 gene that are involved in determining the efficiency of H5 gene transcription in injected frog oocytes.

Firstly, the region from -395 to -174 (relative to the cap site at +1) was found to contain sequences which inhibit H5 gene transcription. This suggests that either some property of this region alone causes inhibition, or that this region binds an endogenous oocyte repressor.

Several other examples of inhibitory regions have been described in various systems. For instance, deletion of an upstream sequence in the sea urchin histone H2A gene produces a two-fold increase in the transcription of this gene in frog oocytes (Grosschedl and Birnstiel, 1980). Other examples were discussed in section 1-3-4(7).

Secondly, the region of the H5 gene from -174 to -85 was found to contain sequences which stimulate transcription of the gene in frog oocytes. The most likely candidates for such sequences are the two GC boxes (1-3-4(2)) located at approximately -160 and -90. The '-90 GC box' is a perfect match to a GC box found to be a high affinity binding site for the protein Sp1 (5' GGGGCGGGGC 3';Kadonaga et al., 1986; 1-4-4(3); figures 2.1 and 5.3). Furthermore, the GC boxes of the HSV TK gene are required for the efficient transcription of this gene in frog oocytes (McKnight et al., 1984; 1-3-4(2)). Also, the GC box was found to be an important promoter element for a chicken H1 gene, in both frog oocytes and HeLa cells (Younghusband et al., 1986).

At this stage, no further characterisation of the inhibitory or stimulatory regions has been carried out using the frog oocyte system.

Also, it is not yet known if the same regions affect H5 gene transcription in other systems or in erythroid cells. However, experiments are currently underway in this laboratory to test these possibilities.

(2) Use of 5' and 3' H5 gene deletions in CSWF co-injection studies

The CSWF effect was obtained with an H5 gene starting at -85, relative to the cap site. This suggests that:

(i) the CSWF effect is not the result of removing the inhibitory effect of the -395 to -174 region (e.g. by a protease destroying a repressor protein);

(ii) the CSWF effect is not due to Sp1 molecules in the fractions, which would interact with the GC boxes at -160 and -90.

As noted in 5-5, the results obtained in CSWF co-injection experiments using mH5/-85 and mH5/-174(delPP) suggest that a region of the H5 gene sufficient to generate the CSWF effect is located between -85 and +313. However, it is still possible that more than one region can produce this effect. For example, one such region may be located between -174 and -85, and another may lie between -85 and +1360. Nevertheless, the 398 bp region from -85 to +313 is a convenient region with which to begin a more detailed analysis of the sequences involved in the CSWF effect.

CHAPTER 6

INVESTIGATIONS INTO THE NATURE OF THE STIMULATORY FACTOR(S) AFFECTING H5 GENE TRANSCRIPTION

CHAPTER 6 : INVESTIGATIONS INTO THE NATURE OF THE STIMULATORY FACTOR(S) AFFECTING H5 GENE TRANSCRIPTION

6-1 Introduction

This chapter describes preliminary work on the nature of the factor(s), present in the CSWFs, which are involved in the trans-stimulation of H5 gene transcription in co-injected oocytes. This investigation has initially focused on two questions:

(1) What is the molecular nature of the factor(s) ?

(2) Are the factor(s) erythroid cell-specific ?

The first of these questions is addressed in sections 6-2 and 6-3, while the second is considered in section 6-4.

6-2 Effect of protein removal on trans-stimulation by the CSWFs

The initial hypothesis considered for the molecular nature of the stimulatory factor(s) was that these factors were most likely to be proteins. This hypothesis was based on findings from other studies, which implicate proteins, particularly DNA binding proteins, in the regulation of transcription (refer to section 1-4 for examples). To test this hypothesis, experiments were carried out in which proteins were removed from the CSWFs and the treated fractions were examined for their trans-stimulation activity in oocyte co-injection assays.

Samples of CSWFs (approximately 6 ug) were incubated with 50 ug of proteinase K (9-2-2) for 30 - 60 minutes, at 37 ^{o}C. The mixture was then subjected to one phenol/chloroform extraction (to remove any residual protein and the proteinase K), followed by one chloroform extraction (to remove all traces of phenol, which would be deleterious to the oocytes). The resultant solution was concentrated (by vacuum dessication) and mixed with a DNA sample for subsequent injection. Approximately 5 - 10 ng of total DNA, plus the treated CSWF, was injected into the nucleus of each of

a batch of 20 - 30 oocytes.

As controls in these experiments, untreated CSWFs and CSWFs handled similarly to the treated fractions, except for the protein removal and extraction steps, were used. As with all of the co-injection experiments, PIB (or BSA in PIB) was also injected, with the DNA, into one batch of oocytes.

The result of one of the protein removal experiments is shown in figure 6.1. A mixture of mH5/-174 DNA and pH1/H2A DNA was co-injected into each batch of oocytes, with either BSA (25 ng per oocyte; track 1), a sample of CSWF II (25 ng ; track 2) or a sample of CSWF II which had been treated to remove protein (as described above; track 3).

It can be seen that both the untreated and treated CSWFs produce the trans-stimulation of H5 gene transcription (shown in figure 6.1 as an increase in the level of H5 transcripts relative to the level of H1 transcripts). It is also apparent that the treated CSWF produces a larger stimulation effect than the untreated fraction. This latter result has been observed several times and may indicate that protein in the CSWFs actually inhibits the stimulation effect to a small degree.

A total of 5 sets of experiments were carried out in which CSWFs treated with proteinase K (and phenol extraction) were found to produce the trans-stimulation effect. Moreover, this has been demonstrated with both CSWFs I and II (CSWF III has not yet been tested in this type of experiment), and with oocytes taken from 4 different frogs.

To ensure that the proteinase K was active in the presence of the CSWFs, samples of CSWFs were incubated with proteinase K, under the same conditions used for the oocyte experiments, and then electrophoresed on SDS-polyacrylamide gels, alongside untreated fractions. At the level of

Figure 6.1 : Effect of proteinase K treatment and phenol/chloroform extraction on the activity of the CSWFs

The result of primer extension analysis of RNA from three batches of injected oocytes is shown. Track 1 represents the injection of a mixture of mH5/-174 DNA, pH1/H2A DNA and bovine serum albumin, as a control. Track 2 represents injection of the DNA mixture with a sample of the second preparation of CSWFs. Track 3 represents injection of the DNA mixture with a sample of the CSWF previously subjected to proteinase K digestion and phenol / chloroform extraction (as described in the text). The H5 and H1 extension products are indicated.


1 2 3
H5
H1

detection of the gels (stained with Coomassie brilliant blue R), the proteinase K was found to completely remove all protein components of the CSWFs.

The results of these experiments suggest that a CSWF protein is not responsible for the trans-stimulation effect, or, alternatively, that the active component of the CSWFs is an extremely protease-resistant protein. This latter possibility seems very unlikely, particularly since this 'resistant' protein must also have the ability to survive the phenol/chloroform extraction step in the treatment described above.

Although it is difficult to show conclusively that a protein in the CSWFs is not involved in the H5 trans-stimulation effect, further supportive evidence for this is presented in the next section.

6-3 Trans-stimulation with a nucleic acid fraction from AEV cell nuclei

The most likely alternative to a protein being the active component of the CSWFs was the possibility that a nucleic acid molecule was involved in the H5 trans-stimulation effect. Gel analysis indicated that the CSWFs did contain both RNA and DNA (the latter probably representing extrachromosomal DNA molecules and breakdown of the chromosomal DNA). Obviously, nucleic acids are functionally active molecules, and research has shown that specific RNA molecules are involved in a wide range of cellular processes; RNA can even act as a true biological catalyst (Zaug and Cech, 1986; see chapter 8 for further discussion).

Two approaches were used to test the hypothesis that nucleic acid was the active component of the CSWFs. Firstly, samples of the CSWFs were treated to remove nucleic acids, and the treated fractions were tested in oocyte co-injection experiments. Secondly, a nucleic acid fraction was prepared from AEV cell nuclei and tested for its ability to produce the H5 trans-stimulation effect.

(1) Removal of nucleic acids from the CSWFs

(a) DNA

Relative to RNA, DNA was thought to be a less likely candidate for the active component of the CSWFs. Firstly, except for its crucial role as an information-carrying molecule, DNA has not been implicated in the wide range of cellular activities now associated with RNA molecules. Secondly, experiments have shown that DNA injected into the cytoplasm of oocytes is not transcribed (Mertz and Gurdon, 1977), suggesting that the DNA does not enter into the nucleus from the cytoplasm. However, CSWFs injected into the oocyte cytoplasm produce full levels of the H5 trans-stimulation effect (chapter 3). [It is of course still possible that small "regulatory DNA molecules" in the CSWFs could enter the oocyte nucleus.]

As a test of the hypothesis that DNA was involved in the CSWF effect, a sample of CSWF II was treated with RNase-free DNase I (9-2-2; a standard amount of CSWF [6 ug of protein] was incubated with one unit of enzyme for 30 minutes, at 37 ^{o}C). Following the treatment, enzyme (and other protein) was removed by proteinase K digestion and phenol/chloroform extraction, as described above. The CSWF sample treated in this way was still found to produce the H5 trans-stimulation effect (a similar result was obtained as for the protein removal result shown in figure 6.1).

This suggests that DNA is not the active component of the CSWFs.

(b) RNA

To test the hypothesis that RNA was responsible for the CSWF effect, a sample of CSWF II was treated with RNase A (9-2-2; the same amount of CSWF as used in (a) was incubated with 20 ug of heat-treated RNase A for 30 minutes, at 37 ^{o}C). Following the treatment, protein was removed by proteinase K digestion and phenol/chloroform extraction. Again,

the CSWF sample treated in this way was still able to generate the H5 trans-stimulation effect.

This result suggested that RNA was not involved in the CSWF effect. However, it was still possible that the RNA in the CSWFs was protected from digestion by secondary structure and/or binding proteins, which would deny access to the RNase molecules. Similar arguments could, of course, also be applied to the DNA in the CSWFs. Clearly, a more direct test of the nature of the active component of the CSWFs was required.

(2) Testing of a nucleic acid fraction from AEV cell nuclei

To determine whether or not nucleic acid was involved in the H5 trans-stimulation effect, a nucleic acid fraction (NAF) was prepared from AEV cell nuclei and used in oocyte co-injection experiments.

Nuclei were made from approximately 5×10^7 AEV cells, by the method described in 9-3-16(i). These nuclei were then incubated with 125 ug of proteinase K for 1 hour, at 37°C, in the presence of 1% SDS. Following incubation, the solution was phenol/chloroform extracted and nucleic acids were purified by ethanol precipitation. After vacuum dessication, the nucleic acids were resuspended in water and stored frozen at -80°C.

Amounts of DNA and RNA in the NAF were estimated by running samples of the fraction on horizontal agarose gels, alongside standards. This indicated that the NAF contained approximately 50 ug of DNA and 100 ug of RNA.

Samples of the NAF were then tested in oocyte co-injection experiments. The result of one such experiment is shown in figure 6.2. Track 1 is the control track and represents the injection of mH5/-174 and pH1/H2A DNA, together with PIB. The H5 and H1 extension products are indicated. Track 2 represents co-injection (using the nuclear co-injection method) of the same mixture of DNAs, together with a sample of the AEV cell

NAF (such that approximately 12.5 ng of AEV cell DNA and 25 ng of RNA were injected into each oocyte). It is clear that the NAF is capable of generating full levels of the H5 trans-stimulation effect. Furthermore, this result has been obtained in a total of 4 sets of injections.

The positive results obtained with the NAF provide strong support for the hypothesis that nucleic acid is involved in the H5 trans-stimulation effect.

It should again be noted that the extension product generated with H5 mRNA from AEV cells is 9 bases longer than that generated with H5 transcripts from injected oocytes (chapter 2, figure 2.2). Therefore, the injection of H5 mRNA (or the H5 gene) from AEV cells cannot be responsible for the trans-stimulation effect.

The NAF experiment described above was part of a series of injections. Another experiment carried out with the NAF in this series is outlined below.

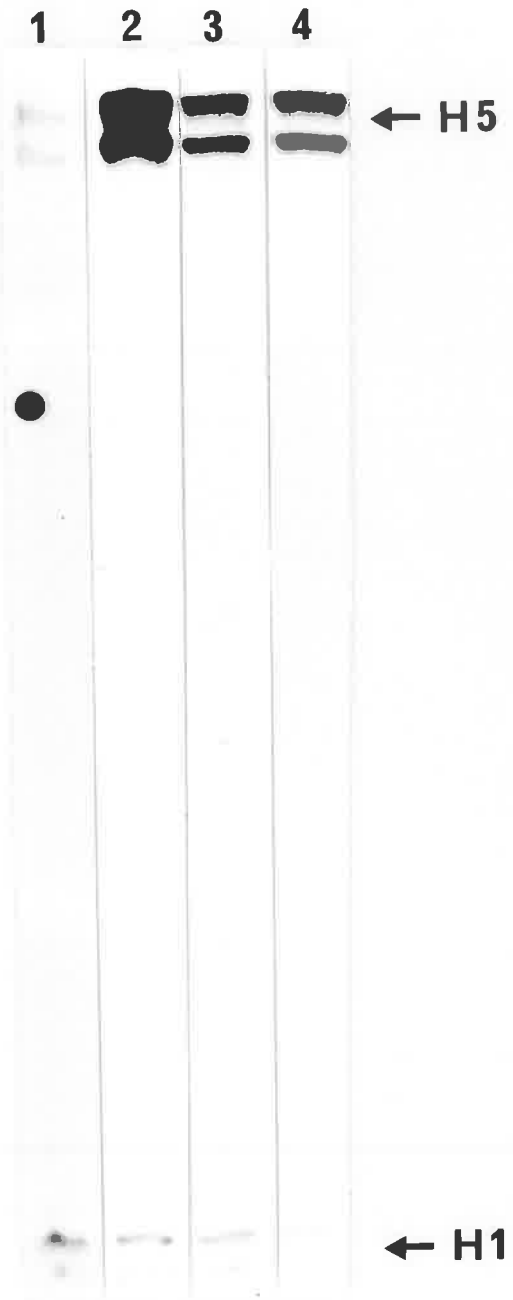
A sample of the NAF (twice as much as used in the experiment described above) was treated with an RNase A/RNase T1 mixture (10 ug of RNase A and 10 units of RNase T1) for 1 hour, at 37 ^{o}C. The enzymes were removed by proteinase K digestion and phenol/chloroform extraction (as previously described). The result obtained following co-injection of the treated NAF is shown in figure 6.2, track 3.

Comparison with the control, track 1, shows that the trans-stimulation effect is still produced by the treated NAF sample. However, when compared with the result obtained with the untreated NAF sample, shown in track 2, it appears that the RNase treatment has significantly reduced the extent of the trans-stimulation effect.

Although this experiment was only a preliminary attempt to

Figure 6.2 : Stimulation of H5 gene transcription with nucleic acid fractions

The result of primer extension analysis of RNA from four different batches of injected oocytes is shown. In each case a mixture of mH5/-174 DNA and pH1/H2A DNA was injected into the nucleus of each of a batch of oocytes, together with either PIB (track 1 - the control), a sample of the AEV cell nuclear nucleic acid fraction (track 2), a sample of this fraction treated with RNase, proteinase K and phenol / chloroform extraction (as described in the text; track 3), or a sample of the T cell nuclear nucleic acid fraction (track 4). The H5 and H1 extension products are indicated.


1
2
3
4
H5
H1

characterise the active component of the NAF, it suggests that an RNA molecule may be involved in the H5 trans-stimulation effect. This RNA molecule also appears to be somewhat resistant to RNase digestion, since treatment with RNase appears to reduce the extent of trans-stimulation generated, but does not eliminate it completely. This could be due to a high degree of secondary structure in the RNA.

The experiments described above suggest an hypothesis whereby an RNA molecule, in the AEV cell CSWFs and NAF, is involved in the trans-stimulation of H5 gene transcription in co-injected oocytes. The rigorous testing of this hypothesis will be the major aim of the next phase of experimentation in this area. This is discussed further, under 'future work', in chapter 8 ('Final Discussion').

6-4 Investigation of the cell type specificity of the stimulatory factor(s)

At the same time as the AEV cell NAF was prepared, a nuclear NAF was also prepared from the chicken T cell line used in the Northern blot experiment, described in chapter 2 (2-5; 9-3-15). The H5 gene is not expressed in these T cells (2-5). As an initial test of the cell type specificity of the factor(s) involved in the H5 trans-stimulation effect, the T cell NAF was used in an oocyte co-injection experiment. Again, this experiment was part of the same series of injections described in 6-3(2).

The result obtained following co-injection of the T cell NAF is shown in figure 6.2, track 4. Comparison with the control track (1) indicates that the T cell NAF is also capable of generating the H5 trans-stimulation effect.

This result can be interpreted in several ways:

(i) the factor responsible for the H5 trans-stimulation effect is found in both the AEV cells and the T cells;

(ii) two different factors, one from each cell type, can produce the same stimulatory effect;

(iii) two different factors, one from each cell type, can each produce a trans-stimulation of H5 gene transcription, but the two factors have two distinct modes of action.

At this stage, no experiments have been carried out to distinguish between these possibilities.

It should be emphasised that although the stimulatory factor may not be erythroid cell-specific, it is not simply a general stimulator of gene transcription, since the trans-stimulation effect is observed with the chicken H5 gene and not with the chicken H1, H2A and H2B genes.

Further discussion on the specificity of the stimulatory factor(s) is given in chapter 8.

6-5 Discussion

This chapter describes some preliminary investigations into the nature and specificity of the factor(s), in the AEV cell CSWFs (and NAF), involved in the trans-stimulation of H5 gene transcription in co-injected oocytes.

Firstly, it was found that removal of protein from the CSWFs, with proteinase K treatment and phenol extraction, did not affect the capacity of the CSWFs to produce the H5 trans-stimulation effect.

Secondly, it was demonstrated that a nucleic acid fraction (NAF) from AEV cell nuclei was also able to generate the trans-stimulation effect. This is consistent with the results of the CSWF protein removal experiments, and suggests that the factor responsible for the trans-stimulation is a nucleic acid.

Thirdly, treatment of the AEV cell NAF with RNase appeared to reduce its capacity to generate trans-stimulation, but did not completely eliminate it. This suggests that an RNA molecule may be involved in the H5

trans-stimulation effect, and that this RNA is protected to some degree from RNase digestion, possibly due to the formation of secondary structure.

With regard to the possibility that the stimulatory factor is an RNA molecule, it is interesting to note that the 3' processing factor (for sea urchin histone H3 mRNA) isolated by Birnstiel's group, using the oocyte co-injection assay, was ultimately found to be a small nuclear RNA - protein complex (snRNP; 1-5-2(4)). Moreover, injection of the purified RNA component of the snRNP into oocytes was also able to bring about the 3' processing event, indicating that oocyte proteins could substitute for sea urchin proteins in the formation of the active snRNP complex. It is possible that a similar explanation may be found for the H5 trans-stimulation effect. This is discussed further in chapter 8.

Finally, it was found that a NAF from chicken T cell nuclei also appeared to be capable of stimulating H5 gene transcription in co-injected oocytes. The similarity of this effect to the effect produced by the AEV cell fractions has not yet been investigated. Again, a further discussion of this result is given in chapter 8.

The experiments described in chapters 2 to 6 of this thesis were directed towards the aim of identifying and characterising regulatory factors involved in the transcription of the chicken H5 gene. These experiments demonstrated that nuclear fractions from chicken erythroid cells (and possibly T cells) can stimulate transcription of the H5 gene when co-injected, with the H5 gene, into Xenopus oocytes. Furthermore, this effect appears to be mediated by an enhancer-like activity. A preliminary characterisation was undertaken of the H5 gene sequences involved in the trans-stimulation effect, and the nature and specificity of the stimulatory factor(s). As previously noted in this chapter, a final discussion of the

work described so far in this thesis, and a consideration of the aims of the next phase of experimentation in this area, are presented in chapter 8.

CHAPTER 7

EFFECT OF H5 PROTEIN ON TRANSCRIPTION IN CO-INJECTED OOCYTES

CHAPTER 7 : EFFECT OF H5 PROTEIN ON TRANSCRIPTION IN CO-INJECTED OOCYTES

7-1 Introduction

The H5 gene trans-stimulation experiments were the major focus of the work carried out using the frog oocyte co-injection assay. However, at the same time, a minor study was also undertaken to investigate the functional properties of H5 protein.

As discussed in 1-6-2, H5 protein levels increase during avian erythroid cell differentiation and maturation, and this is correlated with chromatin condensation and a shutdown of replication and transcription in these cells. This suggests the possibility that H5 protein is involved in the generation and/or maintenance of the repressed state of the mature red cells. At this stage, the functional properties of H5 protein have only been investigated using in vitro systems. These experiments suggest that H5 has a higher chromatin binding affinity than H1, and a greater capacity to promote the formation of higher-order chromatin structures (1-6-2). These properties are consistent with a functional role for H5 protein in the maturing erythroid cell.

However, to properly examine the functional properties of H5 protein, it is necessary to test the effects of this protein directly, in a defined biological system. The frog oocyte provides such a system, since it is an intact living cell, and DNA injected into the oocyte nucleus is assembled into an apparently normal chromatin structure (1-5-2). Furthermore, various amounts of protein and DNA (or RNA) molecules can readily be introduced into the same oocytes.

The experiments described below represent an initial investigation into the activities of H5 protein, utilising the frog oocyte as an assay system. Specifically, this study focused on the question: " Can H5 protein modulate transcription from DNA injected into the frog oocyte nucleus ? "

7-2 Effect of H5 protein on transcription from co-injected DNA

To test for possible effects of H5 protein on transcription from co-injected DNA, use was made of a well-defined plasmid (constructed in this laboratory), containing three chicken histone genes. This plasmid, named p7AT, contains a 7 kb EcoRI fragment of chicken DNA, with an H1, an H2A and an H2B gene, in pAT153 (Sturm, 1985; Sturm et al., 1986). This construct was mentioned in chapter 3 as the source of the plasmids pH1/H2A and pH1/H2B, and it is schematically represented in figure 7.1(a).

An extensive study has been carried out on the transcription of the three histone genes on this plasmid, in Xenopus oocytes (Sturm, 1985; Sturm et al., 1986). This, and the fact that the p7AT histone genes are chicken genes, transcribed in active erythroid cells, made this plasmid particularly suitable for use in oocyte co-injection experiments with chicken H5 protein.

The p7AT DNA + H5 protein co-injection experiment which was carried out involved:

(i) injection of three different amounts (approximately 0.8 ng, 8ng and 80 ng, per oocyte) of purified chicken H5 protein (gift from Dr. F. Shannon) into the cytoplasm of three separate batches of oocytes (20 oocytes per batch);

(ii) injection, 3 - 4 hours later, of approximately 5 ng of p7AT DNA into the nucleus of each oocyte;

(iii) incubation of the oocytes for about 24 hours, followed by isolation of total RNA from each batch; and

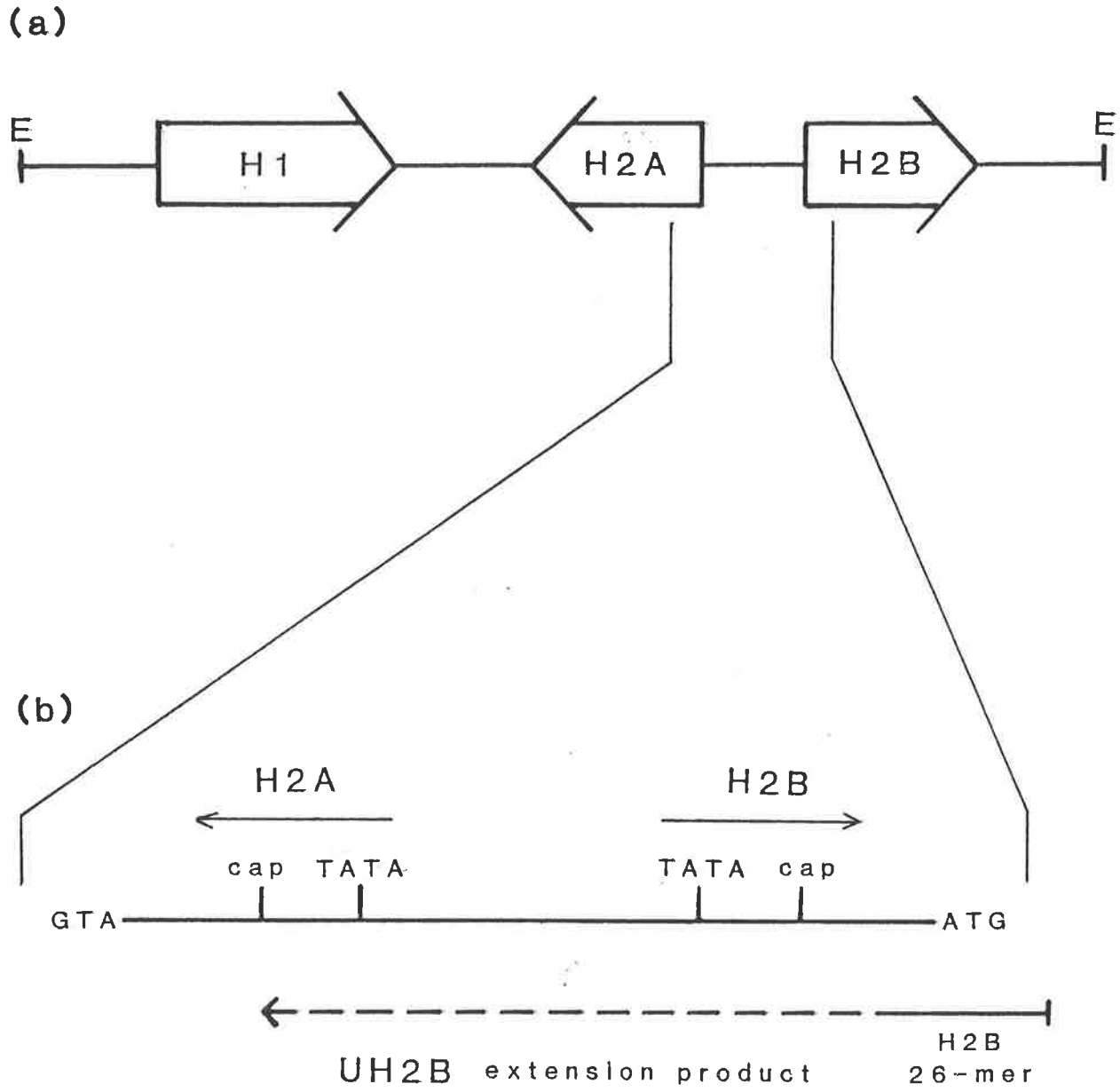
(iv) quantitative primer extension analysis of the oocyte RNA, using the specific histone 26-mers (9-3-12; as used throughout this thesis).

The reason for the cytoplasmic injection of the H5 protein was to allow the protein molecules to migrate to the nucleus and thereby be

Figure 7.1 : The histone genes in p7AT and the UH2B extension product

(a) This shows a schematic representation of the three chicken histone genes in the plasmid p7AT (Sturm, 1985; Sturm et al., 1986). The DNA region depicted is a 7 kb piece of chicken DNA which has been cloned into the EcoRI site of pAT153 (E = EcoRI in the figure).

(b) The H2A-H2B intergene region is depicted. The positions of the TATA boxes, cap sites and ATG initiation codons are indicated. The dashed arrow represents the UH2B primer extension product, generated by reverse transcription from the 3' terminus of the H2B 26-mer.


(a)
E
H1
H2A
H2B
E
(b)
H2A
H2B
cap
TATA
TATA
cap
GTA
ATG
H2B
UH2B extension product
26-mer

available for chromatin assembly of the p7AT DNA, in conjunction with the oocyte histones. Two nuclear injections were avoided because of the potentially low survival of the resultant oocytes. It was predicted that, as is the case for other nuclear proteins, H5 protein would migrate to the nucleus following cytoplasmic injection. This prediction was subsequently confirmed (see section 7-4).

As controls in this experiment, one batch of oocytes was injected with p7AT alone, and another batch was injected with a mixture of histones (approximately 80 ng per oocyte) from CV-1 cells (a monkey kidney cell line), instead of H5 protein.

The result obtained using the H2B 26-mer in primer extension analysis is shown in figure 7.2. Tracks 4 and 5 show the result of extension on RNA from oocytes injected with p7AT + CV-1 histones, and p7AT alone, respectively. Tracks 1 to 3 show the results obtained with increasing amounts of co-injected H5 protein (0.8 ng, 8 ng and 80 ng). Clearly, with the amounts used in this experiment, there is no major effect of H5 protein on the level of H2B gene transcription. With subsequent analysis, this was also found to be the case for H1 and H2A transcription.

However, as shown in figure 7.2, a primer extension product of lower mobility than the H2B products (designated 'UH2B' - see section 7-3), at approximately 305 bases (determined in other experiments which included marker tracks), shows a dramatic response in the three 'H5 protein tracks'. Specifically, the intensity of this band is inversely proportional to the amount of injected H5 protein. In contrast, the injection of CV-1 cell histones (track 4) appears to have had no (or only a very small) effect on the level of intensity of this band.

To confirm these findings, the same experiment as described above was repeated. As before, the injection of H5 protein had no effect on the level

Figure 7.2 : Effect of H5 protein on transcription from p7AT

The result of primer extension analysis, using the H2B 26-mer, of RNA from five different batches of oocytes is shown. p7AT DNA was injected into the nucleus of each oocyte. Track 5 represents the injection of p7AT alone. The other four tracks represent injection of the p7AT DNA following the cytoplasmic injection of the oocytes with either 0.8 ng of H5 protein (track 1), 8 ng of H5 protein (track 2), 80 ng of H5 protein (track 3), or 80 ng of CV-1 cell histones (track 4). The H2B and UH2B extension products are indicated.


1 2 3 4 5
UH2B
H2B

of H2B transcription, but increasing amounts of co-injected H5 protein were again correlated with the 'disappearance' of the UH2B band. Thus, this result was repeatable and was not simply due to variation between batches of injected oocytes.

At this stage, the origin of the UH2B band was unknown, although it had previously been noticed by R. Sturm (in this laboratory) in his studies on p7AT transcription in frog oocytes. The next section discusses the origin and nature of this band, and section 7-4 presents an examination of the H5 protein effect observed in the experiments described above.

7-3 Characterisation of the UH2B band

From R. Sturm's previous results, and from my subsequent experiments, the following conclusions were drawn concerning the origin and nature of the UH2B band in the H5 protein co-injection experiments:

(i) The band represents extension on an RNA polymerase II transcript. This was concluded from an experiment in which p7AT DNA was injected into batches of oocytes with various concentrations of α-amanitin. Following extension with the H2B 26-mer, the pattern observed for the UH2B band was identical to that obtained with the H2B extension product(s). That is, the same concentration of α-amanitin which completely inhibited production of the H2B transcript (10 pg per oocyte) also inhibited the appearance of the UH2B band [Note that Gurdon and Melton (1981) state that 5 pg of α-amanitin per oocyte inhibits RNA polymerase II transcription, whereas RNA polymerase III gene transcription is only inhibited by the injection of 2.5 ng per oocyte.]

(ii) The band represents extension (by the H2B 26-mer) on a transcript, derived from p7AT, after injection into frog oocytes. This was concluded because the band was only present when the H2B 26-mer was used in an extension reaction with RNA from p7AT-injected oocytes. Extension on

uninjected oocyte RNA and RNA from chicken embryos did not result in the appearance of the band. Therefore, the production of the transcript is an artifact of the frog oocyte system.

(iii) The transcript which generates the UH2B band appears to be initiated from a region between the H2A and H2B genes. This was concluded from extension analysis of RNA from oocytes injected with various deletions of p7AT, and with specific, circularised fragments of p7AT. Since the transcript is initiated upstream of the H2B mRNA, and is transcribed from the same template strand as the H2B mRNA, it is referred to as the UH2B transcript ('upstream H2B').

Figure 7.1(b) shows the likely location of the transcription initiation site of the UH2B transcript, shown as the end-point of the UH2B extension product, given that the UH2B product is approximately 305 bases in length. [This assignment of the UH2B cap site assumes that the 305 base extension product does not represent a transcript which initiates further upstream and has one or more introns removed.]

It is interesting to note that the predicted UH2B start site is located at the same position as the H2A transcription initiation site. This suggests that the UH2B transcript may be produced in the oocyte as a result of transcription initiation at the H2A start site. Thus, the two transcripts would be bi-directionally produced from the one point on the DNA template (and, of course, would be read from opposite strands).

A stretch of three 'A' residues is located approximately 25 bp 5' to the UH2B start site (sequence shown in Sturm, 1985 and Harvey et al., 1982). This is the same sequence as the only possible 'TATA box' of the H2A gene and, therefore, may represent the UH2B 'TATA box'. Alternatively, the UH2B transcript may simply be produced as a consequence of H2A transcription initiation (as described above) and specific UH2B promoter

elements may not exist.

The UH2B transcript appears to be initiated approximately 235 bp upstream from the H2B cap site. Upstream initiation sites have also been observed with other genes. For example, in frog oocytes, transcripts from the human beta-globin gene are initiated from -231 and -177, relative to the 'normal' cap site at +1 (Partington et al., 1984).

7-4 Examination of the H5 protein effect

Having identified the 305 base extension product as the UH2B transcript, the H5 protein effect described in 7-2 can be considered in more detail. It appears, from the result presented in figure 7.2, that injection of increasing amounts of H5 protein gives rise to a progressive decrease in the level of the UH2B transcript. The fact that, in vivo, H5 is involved in the packaging of DNA into chromatin, suggests that the protein would exert its effect on the UH2B transcript at the transcriptional level, rather than at the level of transcript stability. Therefore, the result shown in figure 7.2 suggests that injection of H5 protein inhibits UH2B transcription from co-injected p7AT DNA.

It should be noted that although injection of core and H1 histones can inhibit transcription of some injected templates in frog oocytes (Gargiulo et al., 1984), the amount of CV-1 histones used in the p7AT co-injection experiment did not result in inhibition of UH2B transcription. In contrast, injection of the same amount of H5 protein resulted in complete inhibition of UH2B transcription. This suggests that the particular properties of H5 protein were important for the generation of this effect.

To support the hypothesis that H5 protein inhibits UH2B transcription, it was necessary to show that H5 protein was migrating to the nucleus, following cytoplasmic injection. This was shown in the following

experiment:

(i) Purified H5 protein, which had been labelled with ^{125}I (gift from Dr. F. Shannon), was injected into the cytoplasm of each of a batch of 30 oocytes. Approximately 80 ng was injected into each oocyte; this was the largest amount injected in the p7AT + H5 protein experiment, and resulted in essentially complete inhibition of UH2B transcription.

(ii) At three different time points, approximately 30 minutes, 3 hours and 5 hours after injection, a third of the oocytes were removed from incubation at 18 °C and the nucleus of a number of these oocytes was successfully isolated away from the cytoplasm by manual enucleation (Colman, 1984, chapter 10). The nuclei were rinsed several times in modified Barths' saline (9-3-14(iv)), to remove any attached cytoplasm.

(iii) The nuclei obtained for each time point were pooled and counted. Similarly, the cytoplasmic fractions were pooled and counted. The results obtained from this experiment are shown in the following table (c.p.m = counts per minute).

TIME	NUCLEUS or CYTOPLASM	C.P.M.	NO. OF OOCYTES	C.P.M./OOCYTE NUC. or CYTOP.	% OF TOTAL
30 mins.	cytop.	3285	5	657	94.5
	nuc.	192		38	5.5
3 hours	cytop.	1692	6	282	86.8
	nuc.	258		43	13.2
5 hours	cytop.	1687	8	211	84.9
	nuc.	301		38	15.1

After 3 hours, approximately 13% of the total H5 protein present in the oocytes is localised in the nucleus. If no H5 protein was degraded in the 3 hours, this means that approximately 10 ng of H5 protein was contained within the nucleus of each oocyte, after this time. This is about 10 times as much H5 needed to give a 1 : 1 ratio of H5 to the nucleosomes on the injected p7AT DNA. Even allowing for some degradation, it can be seen that a significant amount of H5 protein would be present in the nucleus of the injected oocytes after 3 hours. Therefore, in the p7AT + H5 protein co-injection experiment, a significant amount of H5 protein would have been available for chromatin assembly on the p7AT DNA.

Thus, the result of the labelled H5 protein injections further supports the hypothesis that H5 protein, presumably when assembled into p7AT chromatin in co-injected oocytes, can inhibit UH2B transcription.

7-5 Discussion

The work described in this chapter represents a preliminary investigation into the functional properties of H5 protein, using the Xenopus oocyte as a biological assay system. The results of this investigation are as follows:

(1) Injection of purified H5 protein into the cytoplasm of oocytes, followed 3 to 4 hours later by nuclear injection with p7AT DNA, results in a decrease in the intensity of a 305 base H2B 26-mer extension product (figure 7.2).

(2) This extension product is derived from an RNA polymerase II transcript (the UH2B transcript) which appears to initiate approximately 235 bp upstream from the H2B cap site, in the H2A/H2B intergene region of p7AT (figure 7.1).

(3) Injection of ^{125}I-labelled H5 protein into the cytoplasm of oocytes results in the subsequent nuclear localisation of a significant

amount of the injected protein.

These results suggest that H5 protein inhibits UH2B transcription when assembled into p7AT chromatin, in co-injected oocytes.

Note that injection of increasing amounts of H5 protein produced a progressive decrease in the level of UH2B transcripts. This suggests that the extent of the inhibitory effect of H5 protein is related to the amount of the protein present in co-injected oocytes. This may reflect the situation in maturing avian erythroid cells, since, as discussed above, the progressive shutdown of replication and transcription in these cells occurs as the level of H5 protein increases. Also, in both the erythroid cells and the co-injected oocytes, H5 protein must compete with H1 molecules during chromatin assembly.

It is also interesting to note that H5 protein can inhibit transcription from discrete 'mini-chromosomes' in the co-injected oocytes; large chromatin regions, more suitable for the generation of higher order structures, are not required for this particular effect. Again, this may have significance for the function of H5 protein in avian erythroid cells.

One possible interpretation of the result of the p7AT + H5 protein co-injection experiment is that H5 may have the capacity to inhibit transcription in vivo, and thus, may be directly involved in the progressive shutdown of synthetic activity in chicken erythroid cells. It should be noted, however, that H5 protein did not affect transcription from the H1, H2A or H2B cap sites, in the oocyte co-injection experiment. This may have been related to the amount of H5 protein used. It is possible that higher levels of co-injected H5 protein may have resulted in the inhibition of all transcription from the p7AT DNA.

Further work on H5 protein, using the oocyte co-injection assay, has

been carried out in this laboratory (Madley, 1985). Briefly, this showed that the UH2B inhibition effect was repeatable with oocytes taken from different frogs, and that H5 protein could also inhibit RNA polymerase III transcription (of co-injected 5S RNA genes). This work also demonstrated that H1 protein could partially inhibit UH2B transcription (two-fold), but that under the same conditions, H5 protein completely abolished production of the UH2B transcript.

Approaches which may be undertaken in the future include: investigating the effect of higher levels of H5 protein on transcription from p7AT; verifying that H5 does interact with the p7AT 'mini-chromosomes' (with, for example, electron microscopy studies); and examining the possible effect of H5 protein on nucleosome spacing and positioning, using nuclease digestion techniques.

In conclusion then, although work in this area is only in its initial phase, it appears that the frog oocyte co-injection assay may be very useful in studies on the functional properties of H5 protein.

CHAPTER 8

FINAL DISCUSSION

CHAPTER 8 : FINAL DISCUSSION

This discussion will focus on the major area of experimentation described in this thesis, viz., the identification of transcription factors for the chicken H5 gene. The H5 protein work was discussed in the previous chapter.

8-1 Conclusions and Discussion

Prior to the use of the Xenopus oocyte as an assay system for the identification of H5 gene transcription factors, it was demonstrated that transcription of the H5 gene was accurately initiated after injection into the oocyte nucleus. Subsequent experiments defined upstream regions of the H5 gene involved in determining transcriptional efficiency in the frog oocyte. The region from -395 to -174 (relative to the cap site at +1) was shown to have an inhibitory effect on H5 gene transcription. Conversely, the region from -174 to -85 was shown to have a stimulatory effect on H5 transcription. Two sequences with homology to the GC box are present in this latter region.

Chromatin salt-wash fractions (CSWFs) were isolated from a transformed chicken erythroid cell line (AEV cells). Co-injection of these fractions into oocytes, with the H5 gene and control genes (on separate plasmids), resulted in an increase in the level of H5 transcripts produced, relative to the level of control gene transcripts.

The H2B gene used as a control in the previous experiment was cloned next to the H5 gene, in an M13 vector. Use of this clone in CSWF co-injection experiments resulted in an increase in the level of both H5 and H2B transcripts, relative to transcripts from control genes on separate plasmids. However, when a clone of the H2B gene in M13, without the H5 gene, was used, the level of H2B transcripts was not increased by the CSWFs. These results suggest that:

(i) the CSWFs stimulate H5 gene transcription;

(ii) this effect involves an enhancer-like activity; and

(iii) the effect is mediated by H5 gene sequences.

An initial investigation was carried out into the regions of the H5 gene involved in the trans-stimulation effect. Use was made of various 5' deletions and a 3' deletion, of the H5 gene. The results of these studies suggest that a region from -85 to +313 is sufficient to mediate trans-stimulation by the CSWFs. However, it is possible that more than one H5 gene region can mediate this effect.

A preliminary investigation was also made into the nature of the stimulatory factor(s) present in the CSWFs. It was shown that the activity of the CSWFs is not sensitive to treatment with proteinase K and phenol extraction. Furthermore, a nucleic acid fraction from AEV cell nuclei is also capable of stimulating H5 gene transcription in co-injected oocytes, and the activity of this fraction is partially sensitive to RNase treatment. These results suggest that an RNA molecule may be the active component of the stimulatory AEV cell fractions. If this is the case, it seems likely (on theoretical grounds and following general precedents) that the active RNA molecule would function as part of an RNA-protein complex (RNP).

Aside from the long-standing roles of RNA in the cell, RNA and RNPs are now known to possess other, more recently defined, cellular activities. U1 snRNP (small nuclear RNA-protein complex) is involved in the splicing of introns from RNA polymerase II (RNAP II) gene transcripts (Keller, 1984). U4 snRNP may be involved in 3' end processing of polyadenylated RNAP II transcripts (Berget, 1984; Birnstiel et al., 1985). The factor responsible for the 3' end processing of sea urchin histone H3 gene transcripts in co-injected oocytes was shown to be a snRNP (U7 snRNP;

Birnstiel et al., 1985). Finally, the intervening sequence of Tetrahymena ribosomal RNA can act as a true biological catalyst (Zaug and Cech, 1986).

At this stage, no RNA molecule or RNP has been shown to be involved in the control of transcription initiation. However, it was noted by Galli et al.(1983) that co-injection of a nuclear RNA fraction (from sea urchin embryos) into frog oocytes, with sea urchin histone genes, consistently stimulated transcription of the injected genes. Given the diverse range of cellular processes which involve RNA molecules, it may not be surprising to find RNA or RNPs involved in the initiation of transcription.

If an RNP is involved in the trans-stimulation of H5 gene transcription, then oocyte proteins must be able to form active complexes with the RNA in the nuclear AEV cell nucleic acid fraction, used in oocyte co-injection experiments. Support for this comes from the work of Birnstiel's group, since it appears likely that oocyte proteins form an active complex with naked U7 RNA, when the latter is injected into the oocyte cytoplasm (Birnstiel et al., 1985). However, despite this, it does seem surprising that oocyte proteins would be able to form an active complex with an RNA molecule involved in the transcription of the 'cell type-specific' chicken H5 gene.

Two other points should be made concerning the possibility that an RNA is the active component of the AEV cell fractions. Firstly, the observation was made that the fractions lost activity with repeated freezing and thawing. One possible explanation for this could be that this treatment causes breakdown of the active RNA molecule. Secondly, it is possible that the active RNA is actually an mRNA, which is translated in the oocyte to produce a stimulatory protein. This seems unlikely, since the AEV cell fractions used were all made from nuclei, and (for example) no AEV cell H5

mRNA was detected following injection of the fractions into oocytes and subsequent primer extension analysis.

The H5 trans-stimulation effect was not observed with oocytes taken from some frogs. This variability suggests that an oocyte component may be involved in the effect, and this component varies in its amount and/or properties between the oocytes of different frogs. The stimulatory factor in the AEV cell fractions may interact with this oocyte component, to enhance H5 transcription. An example of this type of interaction has been found in experiments with the adenovirus E1A protein. This protein stimulates transcription from a number of viral and cellular promoters (1-4-4(2)). The results of recent studies, using the adenovirus early E2 promoter, suggest that E1A acts by enhancing the binding of another transcription factor to the promoter region (Kovesdi et al., 1986).

A nucleic acid fraction made from the nuclei of transformed chicken T cells was also shown to be capable of stimulating transcription of the H5 gene in co-injected oocytes. At this stage, the nature of this effect, and its similarity to the effect obtained with the AEV cell fraction, are unknown.

It is possible that a more general transcription factor (in the same category as Sp1) may be responsible for the effect of the T cell fraction, and that the stimulatory factor in the AEV cell fractions is a different, erythroid-specific factor. Alternatively, the AEV cell factor may not be cell type-specific; it may be found in blood cells (and therefore in AEV cells and T cells), or it may be found in a range of different cells. It should be recalled, however, that the AEV cell fractions selectively stimulate H5 gene transcription, and do not affect transcription of closely related chicken histone genes. Therefore, the stimulatory factor is at

least somewhat gene-specific in its action.

If the same stimulatory factor is present in both AEV cells and T cells, it may be that the cell type-specificity of H5 gene expression is regulated at the level of mRNA stability, rather than at the transcriptional level. Alternatively, another feature of the erythroid cells may confer cell type-specificity at the level of H5 gene transcription. For instance, an 'open' chromatin conformation on the H5 gene, allowing access to transcription factors, may only be generated in the erythroid cells. In this regard, it is interesting to note that the H5 gene is sensitive to DNase I in chicken red cells, but not in the chicken T cells used in the co-injection experiments (Hutchison and Weintraub, 1985). Also, it has been shown that the H5 gene is associated with the nuclear matrix in the AEV cells, but not in the T cells (Dalton et al., 1986b).

Finally, it should be noted that both the AEV cells and the T cells are virally transformed cell lines. Albeit unlikely, it is possible that the transformed state of the cells in some way contributed to the results of the experiments described in this thesis. One of the future aims of this work (discussed in the next section) is to confirm the results of the co-injection experiments with fractions isolated from 'normal' chicken erythroid cells.

8-2 Future work

Several aspects of the work described in this thesis will be the subject of future work in this area.

(1) The major aims of the next phase of experimentation will be to characterise and purify the stimulatory factor(s) in the AEV cell fractions. Particular attention will be focused on the hypothesis that an RNA molecule may be involved in the H5 trans-stimulation effect. This research will involve the preparation and testing of RNA fractions from AEV

cell nuclei, and further treatment of AEV cell CSWFs and nucleic acid fractions to remove RNA.

Purification will probably involve column fractionation; H5 DNA affinity columns may be useful in this work. If an RNA molecule is the stimulatory factor, it may be possible to isolate it by cloning procedures.

(2) The gene- and cell type-specificity of the trans-stimulation effect will be further investigated, with the use of different genes in co-injection experiments, and fractions isolated from different cell lines. Also, the nature of the T cell stimulation effect will be examined in more detail.

(3) Nuclear fractions isolated from erythroid cells taken directly from the chicken will be tested to check that the transformed state of the AEV cells is not responsible for the trans-stimulation results.

(4) Further deletions and mutations of the H5 gene will be made, for use in a more detailed investigation of the H5 gene sequences involved in the trans-stimulation effect.

(5) DNA binding studies will be undertaken to determine if the binding of factors to the H5 gene can be correlated with the identification of specific regions of the gene involved in trans-stimulation of transcription.

(6) Experiments are currently underway in this laboratory to determine if the AEV cell fractions can stimulate transcription of the H5 gene in an in vitro system, made from HeLa cell extracts. This would provide an easier assay system, particularly for the testing of fractions from columns used in a purification scheme. Oocyte extracts may also be tested for use as an assay system.

CHAPTER 9

MATERIALS AND METHODS

CHAPTER 9 : MATERIALS AND METHODS

9-1 Abbreviations

Abbreviations were as described in "Instructions to authors" (1978). In addition:

BCIG : 5-bromo-4-chloro-3-indolyl-β-D-galactoside

bisacrylamide : N,N'-methylene-bisacrylamide

ddNTP : dideoxynucleoside triphosphate

DTT : dithiothreitol

IPTG : isopropyl-β-D-thio-galactopyranoside

PIPES : piperazine-N,N'-bis(2-ethnane-sulfonic acid)

PEG : polyethylene glycol

PMSF : phenylmethylsulfonylfluoride

SDS : sodium dodecyl sulphate

TEMED : N,N,N',N'-tetramethylethylenediamine

9-2 Materials

9-2-1 Chemicals and Reagents

All chemicals were of analytical reagent grade, or the highest available purity. Most chemicals and materials were obtained from a range of suppliers, the major sources of some of the more important chemicals and reagents are listed below.

Acrylamide, agarose, ATP, ddNTPs, dNTPs, DTT and bisacrylamide - Sigma

Low melting point agarose - B.R.L.

Mixed bed resin AG 501-X8 (D) - Bio-rad

Urea (ultra pure) - Merck

Chloramphenicol - gift from Parke-Davis

Nonidet P40, formamide, glyoxal and PEG 6000 - BDH

M13 universal primer, γ-^{32}P-ATP, α-^{32}P-dCTP and α-^{32}P-dATP - Biotechnology Research Enterprises of South Australia (BRESA)

9-2-2 Enzymes

Enzymes were obtained from the following sources:

AMV reverse transcriptase - Molecular Genetic Resources

Calf intestinal phosphatase and Ribonuclease A - Sigma

E.coli DNA polymerase I and Klenow fragment - Boehringer Mannheim

BRESA

Proteinase K - Boehringer Mannheim

Restriction endonucleases - Boehringer Mannheim

New England Biolabs

RNase-free DNase I - Promega Biotec

S1 nuclease - Boehringer Mannheim

T4 DNA ligase - New England Biolabs

BRESA

T4 DNA polymerase - BRL

T4 polynucleotide kinase - Boehringer Mannheim

US Biochemicals

9-2-3 Bacterial media

All bacteria, except JM101, were grown in L-broth or on L-agar plates. JM101 was grown in minimal medium, 2 x YT broth and on minimal plus glucose plates.

L-broth : 1% (w/v) amine A, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0

L-agar plates contained L-broth with 1.5% (w/v) bacto-agar

Minimal medium : 2.1% (w/v) K_2HPO_4, 0.9% (w/v) KH_2PO_4, 0.2% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) tri-sodium citrate

Minimal plus glucose plates contained minimal medium, 0.4% (w/v) glucose, 0.0001% (w/v) thiamine and 1.5% (w/v) bacto-agar

2 x YT broth : 1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.0.

All media and buffers were prepared with distilled and deionised water and sterilised by autoclaving, except heat labile reagents, which were filter sterilised.

9-2-4 Bacterial strains and cloned DNA sequences

Bacterial stocks

MC1061 - gift from Dr. R. Harvey

JM101 - gift from Dr. A. Robins

Cloned DNA Sequences

p7AT, pH1/H2A and pH1/H2B - gifts from Dr. R. Sturm

pH52.6 - gift from J. Whiting

H5 gene Bal31 clones - gift from Dr. A. Robins

9-3 Methods

9-3-1 Isolation of plasmid DNA

(i) Growth and amplification of plasmid DNA

A loopful of a glycerol stock of E. coli, containing the plasmid to be grown, was streaked on an L-agar plate (usually supplemented with an appropriate antibiotic to maintain selective pressure for the retention of the plasmid; 30 ug/ml) and incubated overnight at 37 ^{o}C. A single colony was used to infect 5 ml of L-broth (supplemented with antibiotic), which was then grown overnight at 37 ^{o}C, with vigorous shaking, if amplification of the plasmid was intended. If amplification was not required, a 100 ml solution of L-broth was infected with a single colony and the broth incubated overnight with shaking.

To amplify plasmid in the 5 ml overnight culture it was diluted 100 fold into 500 ml of fresh broth (without antibiotic) and grown with

aeration at 37°C to an A_{600} of 1.0, at which time chloramphenicol was added to a final concentration of 150 ug/ml, and incubation continued overnight.

(ii) Large scale isolation

The cells from the 100 ml overnight or the 500 ml amplified culture were harvested by centrifugation (JA-10 rotor, 6000 rpm for 10 minutes). The plasmid DNA was isolated by a modified procedure of the alkaline extraction procedure of Birnboim and Doly (1979).

Cell pellets were resuspended in 4 ml of 15% (w/v) sucrose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 2 mg/ml lysozyme and incubated on ice for 20 minutes. 8 ml of freshly made 0.2 M NaOH, 1% (w/v) SDS was added and gently mixed until the suspension became almost clear and slightly viscous. The solution was then left on ice for a further 10 minutes. 5 ml of 3 M sodium acetate pH 4.6, was added and gently mixed by inversion for a few seconds. The tube was maintained on ice for 30 minutes to allow protein, high molecular weight RNA and chromosomal DNA to precipitate, then centrifuged for 25 minutes at 15,000 rpm (JA-20 rotor) to remove the clot. The supernatant containing supercoiled plasmid DNA was carefully aspirated, avoiding lumps of the precipitate, treated with Ribonuclease A (heat-treated at 80°C for 20 minutes to inactivate deoxyribonucleases; 50 ul of a 10mg/ml stock) for 20 minutes at 37°C, phenol-chloroform extracted, then ethanol precipitated (9-3-3).

After centrifugation, the plasmid DNA pellet was redissolved in 1.6 ml of water and 0.4 ml of 4 M NaCl, 2 ml of 13% PEG 6000 added and the solution was then placed on ice for 1 hour (half of these volumes were used when the nucleic acid from a 100 ml bacterial culture was to be PEG precipitated). The plasmid DNA was collected as a pellet after a 10 minute centrifugation in an Eppendorf microfuge. The PEG supernatant was removed,

the pellet washed in 70% ethanol and then dried in vacuo. The pellet was then resuspended, ethanol precipitated, washed, dried and resuspended in an appropriate volume of water.

The yield of DNA, prepared by this method, was approximately 300 to 600 ug per 500 ml amplified culture and 100 ug per 100 ml culture, as assayed by electrophoresis (9-3-4).

(iii) Miniscreen procedure

Colonies were grown overnight in 2 ml of L-broth plus an appropriate antibiotic at 37°C with continual shaking. The cells were then pelleted by centrifugation for 5 minutes in an Eppendorf microfuge. Plasmid DNA was extracted by the same, but scaled down, procedure as described above except that the PEG precipitation procedure was omitted. The DNA prepared in this way was pure enough to be cut with restriction enzymes.

9-3-2 Isolation of M13 replicative form

A single plaque was toothpicked into 100 ml of 2 x YT broth containing 10 ml of a fresh JM101 overnight culture, grown from a single colony picked from a minimal plus glucose plate. This culture was incubated at 37°C with vigorous aeration for 6 hours. The method of isolation of M13 replicative form from this culture was the same as that for the isolation of plasmid DNA (9-3-1). The yield of DNA prepared in this way was approximately 100ug.

9-3-3 Restriction endonuclease digestions

All restriction endonuclease digestions were performed using the conditions for each enzyme described by the suppliers. ATP (50 uM) was also included if the restricted DNA was to be ligated. A two-fold excess of enzyme generally was used and the reactions were run for an hour, although this time was increased for preparative digestions.

Reactions were stopped by the addition of EDTA pH 7.4 to 5 mM, and protein removed by phenol/chloroform extraction or by the addition of a

quarter volume of urea load buffer (4 M urea, 50% (w/v) sucrose, 50 mM EDTA pH 7.4, 0.1% (w/v) bromo cresol purple). In phenol/chloroform extractions one half volume of phenol saturated with 100 mM Tris-HCl pH 9.0, 5mM EDTA, 50 mM 2-mercaptoethanol, was added, mixed and a half volume of chloroform added. After vortex mixing and centifugation (Eppendorf microfuge, 1 minute; JA-20 rotor 5 minutes at 7,000 rpm) the upper aqueous phase was recovered.

DNA was ethanol precipitated from the aqueous supernatant by adjusting the reaction mix to 0.2 M NaCl or 0.3 M sodium acetate pH 5.5 and addition of 2.5 volumes of nuclease-free ethanol. The DNA was pelleted by centrifugation for 10 minutes in an Eppendorf microfuge or a JA-20 rotor (10,000 rpm). The DNA pellet was washed with 70% nuclease-free ethanol and dried in vacuo before being redissolved in an appropriate volume of water.

9-3-4 Routine gel electrophoresis

(i) Polyacrylamide gel electrophoresis

Electrophoresis of DNA species of less than about 1 kb in length was carried out on vertical 14 cm x 14 cm x 0.5 mm slab gels containing 5-20% acrylamide/bisacrylamide (30:1), which had been deionized with mixed bed resin.

A 15 ml mixture of acrylamide and bisacrylamide in TBE buffer (100 mM Tris-borate, 2.5 mM EDTA) was prepared, 250 ul of 10% (w/v) ammonium persulphate and 12.5 ul of TEMED added, and the solution poured into a gel mould and allowed to polymerise. Gel reservoir tanks contained approximately 1 litre of TBE buffer and gels were pre-electrophoresed at 25 mA for 20 minutes before loading. Samples were dissolved in 10 ul of water and a quarter volume of loading buffer (50% (w/v) sucrose, 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.1% (w/v) bromo cresol purple and 0.1% (w/v) xylene

cyanol) and layered directly into gel slots approximately 1 cm wide. When DNA samples of greater than 10 ug were electrophoresed, the DNA samples were dissolved in a larger volume of water and loading buffer and layered into an appropriately sized gel slot.

All acrylamide gels were electrophoresed at 25 mA until the dyes had moved the desired distance. DNA was visualised under UV light after ethidium bromide staining or by autoradiography (9-3-5) if the DNA contained ^{32}P.

(ii) Agarose gel electrophoresis

Agarose was dissolved in TEA (40mM Tris-acetate, 20 mM sodium-acetate, 1 mM EDTA, pH 8.2) to 0.7-2% (w/v) and cast either in 14 cm x 14 cm x 0.3 cm vertical slab gel templates or on to 7.5 cm x 5 cm microscope slides, for horizontal gels. Vertical gels were electrophoresed between reservoirs each containing 500 ml of TEA at 65 mA, usually for 3 hours. Horizontal gels were submerged in 400 ml of TEA buffer and a current of 125 mA was applied for approximately 20 minutes.

DNA samples were dissolved in 10 ul of water and 2.5 ul of loading buffer (50% (v/v) glycerol, 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.1% (w/v) bromo cresol purple) and loaded directly into gel slots for vertical gels. Samples of 5-8 ul were loaded into the horizontal gel slots.

The DNA was visualised with ethidium bromide as described above (9-3-4(i)).

(iii) Low melting point agarose gel electrophoresis

When a DNA sample was to be recovered from an agarose gel, low melting point agarose was used in place of normal agarose in the gel systems described above. To prevent cracking due to shrinkage, the gel was poured after leaving agarose and the gel mould to equilibriate at 37 ^{o}C. The gel was then allowed to set at 4 ^{o}C and electrophoresis was also carried out

at 4°C.

Prior to loading, the gel was pre-electrophoresed for 10 minutes. Electrophoresis was at similar voltages and for similar times to that for normal agarose gel electrophoresis. The bands were visualised and then cut from the gel for DNA isolation (9-3-7).

9-3-5 Autoradiography

^{32}P-labelled DNA, which had been electrophoresed on polyacrylamide gels was visualised by autoradiography. Gels were covered with a thin sheet of plastic-wrap and a sheet of Fuji X-ray film was placed over the gel enclosed in an Ilford autoradiography cassette and exposed at room temperature for the required amount of time. For detection of low levels of radioactivity, autoradiography was carried out in a cassette with a tungsten intensifying screen, at -80°C. After exposure, the X-ray film was developed, fixed, washed and dried automatically.

When DNA was to be eluted from a gel, one of the glass plates of the gel mould was removed and plastic wrap placed over the gel. Two strips of tape were adhered to the plastic wrap each side of the tracks concerned and spotted with radioactive ink (to act as markers). A sheet of X-ray film was placed on the gel and autoradiographed for the required amount of time. After developing and fixing the film, the radioactive ink spots were lined up with the X-ray film and bands cut from the gel for elution (9-3-6).

9-3-6 Elution of DNA from polyacrylamide gels

The gel slice containing the DNA to be eluted was placed in an Eppendorf tube and 600 ul of TE buffer (10 mM Tris-HCl pH 7.4, 0.1 mM EDTA) was added and incubated overnight at 37°C. The buffer was aspirated from the gel slice and the DNA precipitated with nuclease-free ethanol.

9-3-7 Isolation of DNA from low melting point agarose

The smallest slice of agarose possible, containing the DNA fragment, was placed in an Eppendorf tube. The agarose was melted at 65°C, then 200 ul of 0.2 NET buffer (200 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA) added. This mixture was phenol extracted twice without the addition of chloroform, then once with phenol/chloroform. The aqueous phase was then ethanol precipitated. Approximately 50-70% of the fragment loaded onto the gel was recovered in this way.

9-3-8 Subcloning of DNA fragments into plasmid and M13 vectors

(i) End-filling of DNA fragments and blunt-end ligations

Vector DNA was linearised with a suitable restriction enzyme then dephosphorylated with calf-intestinal phosphatase in a 50 ul reaction mix containing 50 mM Tris-HCl pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnSO_4$ and 0.18 units of enzyme. After a 1 hour incubation at 37°C, the enzyme was heat inactivated at 68°C for 15 minutes in the presence of 0.5% SDS and the protein removed by phenol/chloroform extraction. The linearised dephosphorylated vector was purified from uncut vector by passaging the DNA through a low melting point agarose gel.

Restriction fragments to be subcloned were preparatively isolated from either agarose or polyacrylamide gels. When the DNA fragment had protruding 5' or 3' termini, it was treated with DNA polymerase I Klenow fragment to end-fill or digest back the single-strand regions to blunt-ends. This was done, before purification of the fragment by gel electrophoresis, in a 20 ul reaction mix containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 30 uM of each dNTP and 1 unit of Klenow fragment.

Ligation of insert into vector was done in a 10 ul volume containing 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM ATP and 0.5 units of T4 DNA ligase at 4°C for 16 hours. Sufficient insert to give a three fold

molar excess over vector was generally used. 50 ng of plasmid or 10 ng of M13 vector was normally contained in a ligation mix. Recombinant molecules were transformed into bacteria as described below (9-3-9, 9-3-10).

(ii) Sticky-end ligations

These were performed as described above except 0.1 units of ligase was used in the reaction and incubation carried out at 14 °C for 4-16 hours.

9-3-9 Transformation procedure for plasmid recombinants

A single colony of E. coli strain MC1061 was used to infect 5 ml of L-broth which was then grown overnight at 37 °C with aeration. One ml of the overnight culture was diluted 50 fold in 50 ml of L-broth and grown with shaking to an A_{600} of 0.8. The cells were then rapidly chilled on ice for 30 minutes. The cells were pelleted by gentle centrifugation (HB-4 rotor, 5000 rpm for 2 minutes), washed in a half volume of ice cold 0.1 M $MgCl_2$, then resuspended in one twentieth volume of ice cold 0.1 M $CaCl_2$ and left on ice for at least 1 hour. 200 ul of this cell suspension was mixed with 100 ul of ligation mix diluted in 100 mM Tris-HCl pH 7.4 and left on ice for 30 minutes with occasional mixing. The transformation mix was heated to 42 °C for 2 minutes then returned to ice for 30 minutes. After slowly warming to room temperature, 0.5 ml of L-broth was added and the cells incubated at 37 °C for 30 minutes. After this time 3 ml of L-broth containing 0.7% agar was added to the transformation mix and poured onto an L-agar plate containing an appropriate antibiotic, depending on the resistance carried by the plasmid. The plate was incubated overnight at 37 °C.

9-3-10 Transformation procedure for M13 recombinants

A loopful of E. coli strain JM101, from a minimal plus glucose plate, was used to infect 5 ml of minimal medium which was then grown overnight at

37°C with aeration. The overnight culture was diluted 50 fold into 50 ml of 2 x YT and grown with shaking to an A_{600} of 0.4. The cells were pelleted by gentle centrifugation (HB-4 rotor, 5000 rpm for 2 minutes), then resuspended in one-twentieth volume of ice cold 50 mM $CaCl_2$ and left on ice for at least 1 hour. 200 ul of this cell suspension was mixed with a sample of the ligation mix and left on ice for 40 minutes. The transformation mix was heated to 42°C for 2 minutes. 3 ml of L-broth containing 0.7% agar, 20 ul of BCIG, 20 mg/ml in dimethylformamide, 20 ul of IPTG, 24 mg/ml in water, and 0.2 ml of a JM101 overnight culture diluted 1:5 in 2 x YT broth were then added and the mixture plated directly onto a minimal plus glucose plate. Incubation was overnight at 37°C.

9-3-11 M13 chain terminator sequencing of DNA

(i) Preparation of template

M13 phage plaques were toothpicked into 1 ml of a 1:40 dilution of a fresh JM101 overnight culture (grown in minimal medium) in 2 x YT broth. After incubation at 37°C with vigorous shaking for 5 hours, the culture was centrifuged for 5 minutes in an Eppendorf microfuge. The supernatant was poured into an Eppendorf tube containing 200 ul of 2.5 M NaCl, 20% PEG 6000 and left at room temperature for 15 minutes. The single-stranded M13 phage particles were collected as a pellet after centrifugation for 5 minutes. The supernatant was aspirated and the pellet resuspended in 100 ul of 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, phenol/chloroform extracted and the DNA recovered by the addition of 10 ul of 3 M sodium acetate pH 5.5 and 250 ul of nuclease-free ethanol. The DNA pellet was resuspended in 25 ul of 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA and stored frozen at -20°C.

(ii) Sequencing reactions

Four separate reactions, each specific for one of the bases in DNA,

were used in the sequence analysis of the insert of the M13 single-stranded template. In each of the sequencing reactions, the M13 universal primer was extended in the presence of a different ddNTP such that there was a partial incorporation of each, which resulted in termination of synthesis.

The method described below is for the sequencing of one M13 clone but is readily expanded to allow the concurrent sequencing of 8 - 16 clones.

(a) Hybridization

2.5 ng of universal primer (17-mer) was annealed to 5 ul of M13 single-stranded template (prepared as above) in a 10 ul volume containing 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$ by heating the solution to 70 °C for 3 minutes and then hybridizing at room temperature for 45 minutes.

(b) Polymerisation

1 ul of α-^{32}P-dATP (approximately 4 uCi) was lyophilized, the hybridization mix added, vortexed to dissolve the labelled dATP and then 1 ul of 10 mM DTT added. 1.5 ul of each of the appropriate zero mixes (T° for ddTTP: 10 uM dTTP, 200 uM dCTP, 200 uM dGTP, 5 mM Tris-HCl pH 8.0, 0.1 mM EDTA; C° for ddCTP: 200 uM dTTP, 10 uM dCTP, 200uM dGTP, 5 mM Tris-HCl pH 8.0, 0.1 mM EDTA; G° for ddGTP: 200 uM dTTP, 200 uM dCTP, 10 uM dGTP, 5 mM Tris-HCl pH 8.0, 0.1 mM EDTA; A° for ddATP: 200 um of dTTP, dCTP and dGTP, 5 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and ddNTP solutions (0.1 mM for ddCTP and ddATP, 0.5 mM for ddTTP and ddGTP, each in water) were added together. 2 ul of the zero - ddNTP mixtures were added separately to four Eppendorf "reaction tubes".

0.5 ul of DNA polymerase I, Klenow fragment (1 unit/ul) was added to the hybridization mixture - label - DTT solution. 2 ul of this was then added to each of the four reaction tubes and the solutions were mixed by centrifugation for 1 minute. After 10 minutes incubation at 37° C, 1 ul of dATP chase (500 uM dATP in 5 mM Tris-HCl pH 8.0, 0.1 mM EDTA) was added to

each of the four tubes, mixed by a 1 minute centrifugation and incubated for a further 15 minutes at 37°C.

3 ul of formamide loading buffer (100% formamide, 0.1% (w/v) bromo cresol purple, 0.1% (w/v) xylene cyanol and EDTA to 20 mM) was added to stop the reactions and mixed by a short centrifugation. Samples were boiled for 3 minutes and then loaded onto a sequencing gel.

(iii) Sequencing gels

Products of the dideoxy-chain terminator sequencing reactions were separated by electrophoresis on polyacrylamide gels which included 7 M urea as a denaturant. The gels used were 40 cm x 40 cm x 0.35 mm. A 6% gel, which was normally run, was made in the following way. A 85 ml mixture of acrylamide monomer (20:1, acrylamide to bisacrylamide) in TBE buffer containing 7 M urea, was prepared, 800 ul of 10% (w/v) ammonium persulfate and 65 ul of TEMED added, the mixture poured into a gel mould and allowed to polymerise.

Reservoir tanks contained about 4 litres of TBE buffer and the gels were pre-electrophoresed for 45 minutes at 25 mA. Debris and urea were removed from sample wells prior to loading by flushing with buffer from a syringe. All gels were run at 20-30 mA and kept at high temperature during electrophoresis to facilitate DNA denaturation.

Gels were fixed with 200 ml of 10% (v/v) acetic acid and washed with 3 litres of 20% (v/v) aqueous ethanol. After drying, the gels were autoradiographed overnight at room temperature.

9-3-12 'Kinasing' of synthetic oligonucleotides

Synthetic DNA primers were generously provided by Dr. D. Skingle and S. Rogers.

The histone specific 26 base primers prepared were as follows:

H5 primer:

5' dATGGCCGCCGCTTCACCCCACGGCAG 3'

H2A primer:

5' dAGCGACTGAACACTCAGAGAGCAAAC 3'

H2B primer:

5' dGGCTCGGGCATAGTGGCACAACGCGC 3'

H1 primer:

5' dGGCGGGAGCGGTCTCGGACATCGCGG 3'

Primers were 5' end-labelled with T4 polynucleotide kinase and γ-^{32}P-ATP. Normally 50-100 ng of synthetic primer was kinased in a 10 ul reaction containing 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 5 mM DTT, 7 ul of lyophilized γ-^{32}P-ATP (approximately 35 uCi) and 1 unit of enzyme. The reaction was incubated for 1 hour at 37 ^{o}C, 5 ul of formamide loading buffer was added (9-3-11(ii)b) and the mixture was loaded onto a 20% polyacrylamide gel for purification (9-3-4(i), 9-3-6).

9-3-13 Nick Translation of DNA

10 ul each of α-^{32}P-dATP and α-^{32}P-dCTP were added together and dried in vacuo. The labelled nucleotides were then resuspended in a solution of 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 10 mM beta-mercaptoethanol and 50 ug/ml BSA. To this was added cold dGTP and dTTP to 25 uM each, DNase I to 0.5 ng/ml, DNA (usually 100 ng of a restriction fragment isolated from a low melting point agarose gel) and DNA polymerase I (5 units). The total volume was 20 ul. This mixture was incubated at 14 ^{o}C for 2 hours. Following the incubation period EDTA was added to 10 mM and the reaction mix was phenol/chloroform extracted. Labelled DNA was separated from free label on a Sephadex G-50 column.

9-3-14 Injection of Xenopus oocytes (Gurdon, 1977)

(i) Animals

Xenopus laevis females were obtained from Dr. Ray Harris (South Australian Institute of Technology, Pharmacology Department) or Dr. Keith Dixon (Flinders University, Department of Biological Sciences).

(ii) Frog dissection

Frogs were anaesthetised and the ovary, or part thereof, surgically removed. Alternatively the animal was sacrificed (by pithing and decapitation) and the whole ovary obtained. A frog can be recycled up to four times if only a small portion of the ovary is removed during surgery.

In preparation for dissection the frog was placed in a plastic box containing 0.1% Ethyl-m-aminobenzoate (an anaesthetic) for approximately 20 minutes, removed using plastic gloves, washed with tap water and placed on its back. Alternatively, the frog was equally well immobilized by immersion in an ice water slurry for 20 minutes, placed on its back on an ice tray and covered in ice. The skin was swabbed in 0.5% Hibitane/70% ethanol to remove slime.

Since the ovary of a mature female is the largest organ in the animal, the ovarian lobes are easily removed with forceps through a small incision either side of the ventral mid-line. The required number of lobes were cut off with scissors and the excised lobes placed immediately in modified Barths' saline (9-3-14(vi)). Once the incision had been sutured, post-operative care entailed placing the animal in an angled dish, its nose just out of water, until it revived.

(iii) Oocytes

The excised ovary was rinsed in fresh Barths' saline then teased apart with (grade 5) watchmaker forceps. Clumps of oocytes were kept in Barths' saline, in petri dishes, in a temperature-controlled incubator at 17-21 ^{o}C.

Single oocytes for injection were isolated manually from these clumps, with watchmaker forceps. These single oocytes were kept as for the oocyte clumps. Oocytes were only used from 1 to 5 days following removal from the frog.

(iv) Components of the injection system

Micro-injection needles were made from 100 ul micro-capillaries (BLAUBRAND, intraMARK).

An Agla screw-controlled syringe (Wellcome Australia Ltd.) was connected to the needle by plastic tubing of 1 mm internal diameter. The needle was held and manoeuvred by means of a micromanipulator which gives a 4-5 fold reduction in the movement of the hand in all directions (Micro Techniques (Oxford) Ltd.). The tubing and the needle were filled with medicinal paraffin coloured with Fast Red dye enabling discrimination of the paraffin/aqueous interface.

Injections were carried out under a dissecting stereozoom microscope at a magnification of about 15x. A cold light source was used.

(v) Micro-injection technique

(a) Filling of needle

Usually 2 ul samples of injection solution were brought to the microscope stage on a piece of Parafilm. The tip of the paraffin-filled needle was introduced below the surface of the droplet and a portion of the sample was drawn into the needle by screwing out the syringe.

(b) Preparation of DNA for injection

DNA for injection was prepared by the method described in 9-3-1 and 9-3-2, ethanol precipitated several times (all phenol must be removed), and resuspended in a buffer consisting of 88 mM NaCl, 10 mM Tris-HCl pH 7.4. DNA was injected into the nucleus at a concentration of 200-400 ng/ul in a

volume of 25-50 nl.

(c) Nuclear injection

Because the oocyte is not transparent the nucleus cannot be seen. This is only a minor problem as the nucleus occupies a consistent position in the oocyte and is readily injected with the following method.

Each oocyte was transferred from the petri-dish to a dry microscope slide (3-6 per slide) with a wide-mouthed pasteur pipette. Excess fluid was removed with the pasteur pipette as excessively wet oocytes were difficult to grasp with forceps. The slide was then transferred to the microscope stage.

Looking through the microscope the oocytes were individually manoeuvred with forceps and the end of the needle so the animal hemisphere was orientated towards the needle. The needle was inserted at right angles to the oocyte equator exactly over the apex of the animal pole. The needle was inserted about a quarter the depth of the oocyte. At the same time the oocyte was steadied with forceps. Once the needle had been positioned the syringe screw was turned to deliver the required volume by focusing on the calibrated shaft and watching the paraffin/aqueous meniscus. The oocyte was again grasped during needle withdrawal. The group of injected oocytes was then washed off the slide into another petri dish filled with fresh Barths' buffer.

A batch of 20 - 30 oocytes was usually used per DNA template injected. After injection, the oocytes were generally incubated for 24 hours, in modified Barths' saline, at 17 - 21 ^{o}C.

(d) Co-injection

Two methods were employed to introduce DNA, together with proteins or nuclear extracts, into oocytes.

One method involved mixing together the molecules to be introduced and injecting this mixture into the oocyte nucleus. The other method involved injection of the proteins or nuclear extracts into the oocyte cytoplasm (25-50 nl was injected into the vegetal pole) and injection of DNA into the nucleus of the same oocytes.

Proteins or nuclear extracts were usually injected in 'protein injection buffer' (PIB), containing 20mM Tris-HCl pH 7.4, 100mM NaCl, 5mM $MgCl_2$, 1mM EDTA, 0.1mM EGTA, 0.5mM DTT.

(vi) Modified Barths' saline

Modified Barths' saline was made by mixing together 4 stock solutions:

Solution A:	NaCl	206 gm
	KCl	3.0 gm
	$NaHCO_3$	8.1 gm
	Trizma base	73 gm

Made to one litre and pH to 7.6 with concentrated HCl.

Solution B:	$MgSO_4.7H_2O$	10.1 gm/500 ml
Solution C:	$Ca(NO_3)_2.4H_2O$	3.9 gm
	$CaCl_2.H_2O$	3.0 gm

made to 500 ml.

Solution D:	Penicillin	10 mg/ml
	Streptomycin	10 mg/ml

Solutions A,B and C were stored at 4°C. Solution D was stored frozen at -20°C. To make the final solution, 25 ml of A was added to 954 ml of water, followed by the addition of 10 ml of B, 10 ml of C and 1 ml of D.

9-3-15 Cell culture

An AEV-transformed chicken erythroid cell line (ts34 AEV LSCC HD3 : Beug et al., 1982) and a Marek's Disease Virus-transformed chicken T cell line (Akiyama and Kato, 1974) were both grown, in suspension, in DMEM with

10% foetal calf serum and 2% chicken serum. RSV-transformed chicken fibroblast cells were grown attached, in DMEM with 5% foetal calf serum, 10% tryptose phosphate broth (Flow Laboratories) and 1% DMSO.

9-3-16 Isolation of chromatin salt-wash fractions

The following method was used to isolate chromatin salt-wash fractions from cells grown in culture. All procedures were carried out at 4 ^{o}C and all solutions contained 1 mM PMSF to prevent proteolysis.

(i) Nuclei isolation

The cells were spun down, washed in phosphate- or tris-buffered saline, and resuspended in 20 volumes of 1% Triton X-100, 10 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF. Cells were lysed with homogenisation by a loose - fitting (0.13 - 0.15 mm) Teflon pestle in a glass vessel (by hand).

The homogenate was layered onto twice the volume of 30% sucrose, 10 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF and centrifuged at 3500 rpm (approx. 2000 x g) for 15 minutes in a Sorvall HB-4 rotor, to pellet the nuclei.

(ii) Chromatin isolation

The nuclear pellet was resuspended in 50 volumes of a pH 6.5 solution of 80 mM NaCl, 20 mM EDTA, 2 mM EGTA, 1 mM PMSF and homogenised as in (i), thoroughly. Chromatin was pelleted by centrifugation at 5500 rpm (approx. 5000 x g) in a Sorvall HB-4 rotor for 15 minutes. This procedure was repeated twice. The final chromatin pellet was either stored at -20 ^{o}C or used to isolate salt-wash fractions.

(iii) Isolation of salt-wash fractions

A solution containing 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 1 mM PMSF and the required concentration of NaCl (150 mM - 2M)

was added to the chromatin pellet and salt extraction of chromatin-associated proteins was allowed to occur at 4°C, for 30 minutes to 1 hour, with regular mixing. Following this, the mixture was layered onto an equal volume of 10% sucrose in the salt-wash buffer and centrifuged in a Beckmann SW41 rotor at 23,000 rpm for 30 minutes.

The chromatin salt-wash fractions were taken off, dialysed against water and concentrated by vacuum dessication or freeze-drying. The fractions were stored frozen at -80°C in a buffer containing 20 mM Tris pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM EGTA and 0.5 mM DTT (PIB; 9-3-14(v)(d)).

9-3-17 Isolation of Xenopus oocyte and cultured cell RNA

(i) Xenopus oocyte RNA (Probst et al., 1979)

The oocytes were squashed in a loose-fitting glass homogenizer in a solution containing 10 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$, 10 mM NaCl, 1% SDS and 0.5 mg/ml proteinase K (0.5 ml of this solution was used per batch of oocytes). After incubation at 37°C for 30 minutes, EDTA was added to 10 mM and the mixture was extracted two or three times with phenol/chloroform. The aqueous phase was made to 0.2 M NaCl and the oocyte RNA precipitated by the addition of 2.5 volumes of ethanol. RNA was stored in water at -80°C. Oocytes usually yielded 4-5 ug of total RNA each. They were stored dry at -80°C if the RNA extraction was not carried out immediately after incubation.

(ii) Cultured cell RNA

Total cytoplasmic RNA from cells grown in culture was isolated by the following method. The cells were spun down, washed twice in phosphate-buffered saline and resuspended in a solution containing 0.5% NP40 (Nonidet P40), 10 mM Tris-HCl pH 8.0, 10 mM NaCl and 10 mM EDTA. Cells were lysed by leaving them in this solution on ice for 15 minutes, with regular

mixing. The nuclei were then pelleted by centrifugation in a Sorvall HB-4 rotor at 13,000 rpm for 15 minutes. RNA was isolated by phenol/chloroform extraction and ethanol precipitation, and was then stored frozen in water, at -80°C.

9-3-18 Separation of poly A^+ and poly A^- RNA on poly U-sepharose

A 1 ml poly U - sepharose column was initially sterilised with 'elution' buffer (90% formamide, 1 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.2% SDS) and then equilibrated with 'application' buffer (0.5 M NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.5% SDS). The RNA sample was made to 10 mM Tris-HCl pH 7.4, 0.5% SDS, heated to 65°C for 5 minutes, snap chilled and finally made to 0.5 M NaCl.

The sample was applied to the column, followed by the addition of a few mls of 'application' buffer. The initial A^- fraction was collected and then re-applied to the column. A^- RNA was then collected after flushing the column through with 10 mls of 'application' buffer. A^+ RNA was collected following addition of 5 - 10 mls of 'elution' buffer to the column, and the RNA in each fraction was subsequently recovered by ethanol precipitation.

9-3-19 Northern hybridisation analysis of RNA

An RNA sample to be analysed was directly dried in vacuo or ethanol precipitated, and then resuspended in 10 ul of deionised 100% formamide. The sample was then incubated at -80°C for 3 minutes. Following this, 8 ul of 20 mM sodium phosphate solution pH 6.5 (made by dissolving Na_2HPO_4 in water and bringing to pH 6.5 with phosphoric acid) and 3 ul of deionised glyoxal (40%) were added and the resulting mixture was incubated at 50°C for 15 minutes. Finally, 4 ul of loading buffer (50% glycerol, 10 mM sodium phosphate solution pH 6.5, 0.4% bromocresol purple) was added and the

sample was loaded onto a 1 - 2% agarose gel. The gel was run in 10 mM sodium phosphate buffer pH 6.5, at approximately 60 mA, with buffer recirculation.

When the gel had run the desired distance, it was removed from the glass plates and placed on sheets of Whatman 3MM paper (in a tray) which had been wet with 20 x SSC (1 x SSC = 150 mM NaCl, 15 mM Na citrate). Plastic wrap was used to cover the 3MM, leaving only the gel exposed. A sheet of nitrocellulose, previously soaked in 20 x SSC, was placed on the gel and covered with 3MM and a stack of paper towels. A heavy weight was placed on top and the transfer of RNA from the gel to the filter was allowed to take place overnight.

Following transfer, the filter was baked under vacuum at 80 ^{o}C for 2 hours. Glyoxal was removed by placing the filter in a 'just boiled' solution of 20 mM Tris-HCl pH 8.0 and allowing the solution to cool to room temperature. Prehybridisation was carried out at 42 ^{o}C, overnight, in 50% formamide, 5 x SSC, 50 mM KPO_4 pH 6.5, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA and 100 ug/ml sonicated salmon sperm DNA. Subsequent hybridisation with labelled probes was also carried out at 42 ^{o}C, overnight, in the same buffer. The filter was initially washed in 2 x SSC, 0.1% SDS and then in 0.1 x SSC, 0.1% SDS. All washes were done at room temperature or at 65 ^{o}C. The filter was then covered in plastic wrap and exposed to x-ray film.

9-3-20 S1 nuclease analysis of RNA (Berk and Sharp, 1977)

For 3' end analysis, 5' overhang ends of the appropriate restriction fragments were end-filled using DNA polymerase I Klenow fragment (9-3-8(i)) or T4 DNA polymerase, in the presence of α-^{32}P-dNTPs (9-3-8(i)).

Restriction fragments were 3' end-labelled by T4 DNA polymerase using the following method. DNA was added to a 20 ul reaction mixture containing

33 mM Tris acetate pH 7.9, 66 mM potassium acetate, 10 mM Mg acetate, 0.1 mg/ml BSA and 0.5 mM DTT. An appropriate amount of enzyme was added and the mixture was incubated at 37°C for the time needed to remove the required number of nucleotides from each 3' end. Following this, the mixture was added to lyophilised α-^{32}P-dATP and α-^{32}P-dCTP and cold dTTP and dGTP were added to 30 uM each. Polymerisation was allowed to occur at 37°C for 30 minutes. The reaction was stopped by the addition of EDTA to 20 mM and subsequent phenol/chloroform extraction. The labelled DNA was recovered by ethanol precipitation. (Note: at an enzyme to DNA ratio of 1.25 units to 1 ug DNA, approximately 20 nucleotides are excised from each end of the fragment, per minute.)

Probes were also prepared by labelled extension of the M13 universal primer on suitable M13 subclone inserts, as follows. Primer was hybridised with the M13 subclone as for sequencing reactions (9-3-11(ii)(a)). The 10 ul annealing mix was added to lyophilised α-^{32}P-dATP and α-^{32}P-dCTP, followed by the addition of cold dTTP and dGTP to 75 uM each, DTT to 0.5 mM and DNA polymerase I, Klenow fragment (2 units). The 20 ul mixture was incubated at 37°C for 15 minutes to extend the hybridised primer molecules. This reaction was 'chased' by the addition of 3 ul each of cold 0.5 mM dATP and dCTP and a further 15 minute incubation at 37°C. The labelled, double - stranded DNA fragment was isolated from M13 DNA by digestion with appropriate restriction enzymes and purificaiton by acrylamide gel electrophoresis.

For S1 analysis, both double-stranded and single-stranded probes were used, though the single-stranded fragments proved to be better reagents. To prepare single-stranded probes, labelled double-stranded restriction fragments were heat denatured and annealed with an excess of appropriate

M13 single stranded DNA, in a buffer containing 100 mM NaCl, 20 mM Tris-HCl pH 7.4, 0.1% SDS at 65°C, to remove the unwanted hybridising strand. The unhybridised strand was then purified by electrophoresis on a 6% non-denaturing polyacrylamide gel (9-3-4(i)).

Hybridisation reactions were carried out in a volume of 30 ul. The reaction mix contained 80% formamide, 40 mM PIPES pH 6.4, 1 mM EDTA, 400 mM NaCl and incubations were at approximately 50°C for double standed probes and 30°C for single stranded probes, for 12-16 hours. Annealing reactions were diluted with 300 ul of ice-cold S1 buffer (200 mM NaCl, 2 mM $ZnSO_4$, 50 mM sodium acetate pH 4.6) and 1,000-2,500 units of S1 nuclease were added. Digestions were at 37°C for 1 hour; the mixture was then phenol/chloroform extracted, nucleic acids were precipitated with ethanol, dissolved in formamide loading buffer and electrophoresed on 6% sequencing gels (9-3-11(iii)) prior to exposure.

9-3-21 Primer extension analysis of RNA (McKnight et al., 1981)

1 ng of each primer, 5' end-labelled (9-3-12), was separately or together added to oocyte RNA and ethanol precipitated. The pellet was resuspended in 10 ul of 200 mM NaCl, 10 mM Tris-HCl pH 8.3, heated to 70°C for 3 minutes then allowed to anneal at 42°C for 1 - 3 hours. Following hybridisation, the samples were incubated with reverse transcriptase (1 hour, 42°C) in a volume of 34 ul containing 60 mM NaCl, 10 mM Tris-HCl pH 8.3, 10 mM DTT, 500 uM dNTPs, 10 mM $MgCl_2$ and 8 units of enzyme.

Extension products were ethanol precipitated, washed in 70% aqueous ethanol and dried in vacuo. After resuspension in 5 ul of formamide loading buffer the extended primers were electrophoresed on 6% sequencing gels (9-3-11(iii)) and detected by exposure to X-ray film.

9-3-22 Containment facilities

All manipulations involving recombinant DNA were carried out in

accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

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