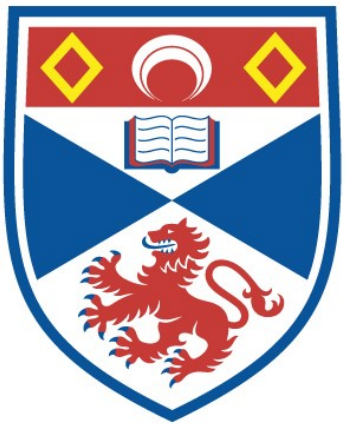
# THE ACTIVITIES OF PROTEIN KINASE CK2 IN OOGENESIS OF `XENOPUS LAEVIS'

Alexander J. Llinas

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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# The Activities of Protein Kinase CK2 in Oogenesis of *Xenopus laevis*

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# Abstract

Protein kinases play important roles in regulating cellular functions in many organisms. This work deals specifically with the protein kinase CK2 (casein kinase II) and its role in regulating the activity of proteins involved in oocyte development in *Xenopus laevis*. Protein kinase CK2 is a tetrameric enzyme containing two catalytic subunits (α and α') and two identical regulatory subunits (β) which forms the holoenzyme. CK2 phosphorylates many different proteins involved in many aspects of cellular functions. It phosphorylates serine and threonine sites and is considered to be a ubiquitous enzyme, expressed at different levels in different cell types.

In this study CK2 activity was characterized in material from two sources: from isolated nuclei and messenger ribonucleoprotein (mRNP) particles from *Xenopus* oocytes. cDNAs expressing both the α and the β subunits were cloned and antibodies were raised against the fusion protein containing the α-subunit. The main objective of this study was to determine the effects that CK2 had on proteins involved in oocyte development.

The interaction of CK2 with a protein known as histone deacetylase was studied in depth to determine how phosphorylation might influence its function and cellular compartmentalisation. Specifically, phosphorylation by CK2 is shown to improve the kinetics of nuclear import, and the interaction of histone deacetylase with α-importin, a well-established nuclear transport protein, is revealed to be dependent on the phosphorylation state of histone deacetylase.

Another aspect of this work is related to the association of CK2 with mRNP particles in the cytoplasm. mRNP particles function as long term storage units for mRNA to be used during oocyte maturation and early embryogenesis. It has been postulated that a protein kinase associated with these particles plays a role in controlling the binding of mRNA to proteins involved in translation repression (mRNA masking proteins). This study lends support to that theory, and the possible effects of CK2 phosphorylation on the masking "Y-box" proteins are discussed.

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# Abbreviations

| | |
|---|---|
| A | Absorption |
| A+ | Polyadenylated |
| Amps | Amperes |
| AMPS | Ammonium persulfate |
| ATP | Adenosine triphosphate |
| cAPK | cAMP-dependent protein kinase |
| CAT | Chloramphenicol acetyl-transferase |
| CBB | Column binding buffer |
| cdk | Cyclin-dependent kinase |
| CDK2 | cyclin-dependent kinase 2 |
| cDNA | Complimentary DNA synthesised from mRNA |
| Ci | Curie |
| CIP | Calf intestinal phosphatase |
| CK2 | Protein kinase CK2 (casein kinase II) |
| CKα | Protein kinase CK2 alpha subunit |
| CKβ | Protein kinase CK2 beta subunit |
| cpm | Counts per minute |
| CSD | Cold-shock domain |
| CTP | Cystidine triphosphate |
| DAB | 3,3'-diaminobenzidine tetrahydrochloride |
| $dH_20$ | Distilled water |
| dATP | Deoxy adenosine triphosphate |
| dCTP | Deoxy cytidine triphosphate |
| DEPC | Diethylpyrocarbonate |
| DEAE | Diethylaminoethyl |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonucleic acid nuclease |
| dpm | Disintegrations per minute |
| DRB | 5,6-dichloro-1β-D-ribofuranosylbenzimidazole |
| DTT | Dithiothreitol |

| | |
|---|---|
| ECL | Enhanced chemiluminescence |
| EDC | carbodiimide |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FGF-2 | Basic fibroblast growth factor-2 |
| FCS | Fetal calf serum |
| fig. | figure |
| FITC | Fluorescein isothiocyanate conjugated anti-rabbit IgG |
| FRGY2a/b | Two Y-box proteins binding to mRNA (p60, p56) |
| GST | Glutathione S-transferase |
| GTP | Guanosine triphosphate |
| GV | Germinal vesicle (nucleus) |
| GVB | Germinal vesicle breakdown |
| ΔH | GST-HDΔH fusion protein, subclone of maternal histone deacetylase |
| HAT | Acetyltransferase |
| HDAC | Histone deacetylase |
| HDACm | *Xenopus* maternal expressed histone deacetylase |
| HEPES | N-[2-Hydroxyethyl] piperazine-N'[2-sulphonic acid] |
| HRP | Horseradish peroxidase |
| hrs | hours |
| IgG | Immunoglobulin |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| kb | Kilo bases |
| kDa | Kilo Dalton (1 Dalton = mass of 1 hydrogen atom) |
| λ | Lambda |
| LB | Luria-Bertani Medium |
| (m/μ/n/p) | (milli/micro/nano/pico) |
| MAPK | (Mos-mediated) mitogen-activated protein kinase |
| min | minutes |
| MMK | MAPK kinase |
| MOPS | 3[N Morpholino] propanesulphonic acid |
| MPF | Maturation-promoting factors |

| | |
|---|---|
| mRNA | Messenger ribonucleic acid |
| mRNP | Messenger ribonucleoprotein |
| NES | Nuclear export signal |
| NP-40 | Nonidet P-40 |
| NLS | Nuclear localization signal |
| NPC | Nuclear pore complex |
| NTF2 | Nuclear transport factor-2 |
| $^0$C | Degrees centigrade |
| OD | Optical Density |
| ORF | Open reading frame |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| pfu | Plaque-forming unit |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| ΔR | GST-HDΔR fusion protein, subclone of maternal histone deacetylase |
| ΔR/ΔH | GST-HDΔR/ΔH fusion protein, subclone of maternal histone deacetylase |
| RB | Retinoblastoma |
| Riboblot | Binding of riboprobe to proteins transferred from SDS-PAGE to nitrocellulose |
| Riboprobe | *in vitro* labelled RNA probe |
| RNA | Ribonucleic acid |
| RNase | Ribonucleic acid nuclease |
| SDS | Sodium Dodecyl Sulphate |
| SN | Supernatant |
| SN10 | Supernatant from a 10,000 g spin |
| SN100 | Supernatant from a 100,000 g spin |
| SSC | Standard sodium citrate |
| TAE | Tris-acetate ETDA buffer |
| TBE | Tris-borate ETDA buffer |

| | |
|---|---|
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline with Tween-20 |
| TCA | Trichloroacetic acid |
| TEMED | N,N,N',N'-tetramethylenediamide |
| Tween-20 | Polyoxyethylene-sorbitan monolaurate |
| UV | Ultra violet |
| ΔV | GST-HDΔV fusion protein, subclone of maternal histone deacetylase |
| V | Volts |
| YB | Y-box |
| YB proteins | FRGY2a and FRGY2b (60 and 56 kDa) |

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# Chapter 1: Introduction

## 1.0 Protein Kinases

The activity of protein phosphorylation by kinases is an important control mechanism for the activation and repression of transcription, translation and cellular transport. Many types of protein kinases exist, which have many different substrates and regulatory cofactors. The importance of protein kinases as regulators is well known and widely studied, especially in signal transduction and cascade events. In general, protein kinases are regulating enzymes that function as reversible covalent modifiers; they add phosphate groups at specific threonine, serine, or tyrosine residues (Edelman *et al.*, 1987). They generally use ATP as the source of these phosphates, but some can also use GTP. This phosphorylation can cause steric changes to the substrate which may either expose or hide the active site, thus increasing or inhibiting its activity. Modulation of activity by phosphorylation may occur through the binding of other stimulating or inhibiting proteins (Edelman *et al.*, 1987).

This study is specifically concerned with the protein kinase CK2 (Casein-Kinase-II) and its regulatory controls in the early development of *Xenopus laevis*. Its expression and activity, as well as its specific function has to some extent been studied in the developing oocytes, but more work is required. The activity of the enzyme in phosphorylating important substrates, such as histone deacetylase and mRNP complexes, has been the major focus of this work. The function of CK2 is not as well defined as that of the other kinases and it has been implicated in many different mechanisms. Its functions are so diverse and ubiquitous that many roles have been postulated, some of which are very different, but none of these roles have been completely explained. The importance of CK2 as a regulatory enzyme is becoming more apparent and will merit more attention.

## 1.1 Protein Kinase CK2

*1.1.1 Structure of CK2:* In many protein kinases a catalytic domain which binds ATP and the substrate to be phosphorylated has been identified. The ATP binding domain in the enzyme CK2 (α subunit) is located near the amino terminus and consists of the amino acid residues 46 G R G K Y S E V 53 (Hanks and Quinn, 1991). Centrally located, usually at Asp 184, is where the transfer of the phosphate group to the substrate takes place. At the carboxy-terminus of the catalytic domain is thought to be located the substrate binding site consisting of the residues 156 D V K P H N 161, but this is still under investigation (see figure 1). The clustering of the basic residues around this acidic region is supposedly very important for interaction with the acidic regions of potential substrates (Kemp and Pearson,1990). The sequence required for CK2 phosphorylation is a serine or threonine site followed by any two non-basic residues and ending with a aspartic acid or glutamic acid residue (S/T X X D/E). These sites are usually surrounded by numerous acidic residues (Pinna, 1990).

```
MSGPVPSRAR   VYTDVNTHRP   RDYWDYESHV 30
VEWGNQDDYQ   LVRKLGRGKY   SEVFEAINIT 60
NNEKVVVKIL   KPVKKKKIKR   EIKILENLRG 90
GPNIITLADI   VKDPVSRTPA   LVFEHVNNTD 120
FKQLYQTLTD   YDIRFYMYEI   LKALDYCHSM 150
GIMHR         VMIDHEHRK   LRLIDWGLAE 180
FYHPGQEYNV   RVASRYFKGP   ELLVDYQMYD 210
YSLDMWSLGC   MLASMIFRKE   PFFHGHDNYD 240
QLVRIAKVLG   TEDLYDYIDK   YNIELDPRFN 270
DILGRHSRKR   WERFVHSENQ   HLVSPEALDF 300
LDKLLRYDHQ   TRLTAREAMD   HPYFYPIVKD 330
QSRMAALICP   VAAHPSVAPV             350
```

**Figure 1: CK2α Functional Domains:** Blue = Region of ATP Binding
Green = NLS
Red = Autophosphorylation Site
= Substrate Recognition sequence

Protein kinase CK2 is a serine/threonine phosphorylating tetrameric enzyme, consisting of two α-subunits and two ß-subunits, which is found in abundance in both

the cytoplasm and the nucleus. However, it is thought that CK2 has roles primarily in the nucleus, having a less active role in the cytoplasm (Krek and Nigg 1992). It can use both ATP and GTP as cosubstrates, with almost equal efficiency (ATP: $K_m$~10mM, GTP: $K_m$~30mM)(Edelman *et al.*, 1987). CK2 is found in many types of tissues throughout the animal kingdom. The highest levels of the enzyme are generally seen in dividing cells, both normal and transformed. The human form of the α subunit gene has recently been completely characterized. The gene spans 70 kb and consist of 13 exons, all of which follow the gt/ag rule in defining the boundaries between intron and exons. The size range of the exons are from 51 to 2960 bp in length, and introns range from 527 to 34000 bp in length. The translation start site has been located in Exon 2, and the stop codon in Exon 13. Promoter activity was detected in the region ranging from -256 to 144, and it seems to show features of a house keeping promoter due to the lack of a TATA box, and the presence of a CpG island, and GC boxes (Winkner *et al.*, 1998). The genes that encode the enzymatic subunits in yeast are *CKA1*, *CKA2*, *CKB1* and *CKB2*. This is different from *Xenopus,* which contains only one β subunit gene (CKB), instead of the two seen in yeast. Gene deletion experiments have shown that deletion of the *CKA1* and *CKA2* are lethal, but deletion of *CKB1* and *CKB2* are not (Glover, 1998). These genes produce the subunits that make up the functional enzyme, and the control of their expression is still unclear. The two α-subunits (α-42kDa and α'-38kDa) are catalytically active, while the two ß subunits (25kDa) act as regulatory subunits by increasing the catalytic activity when bound (Allende and Allende, 1995). The α and α' subunits have extensive sequence identity with one another, about 90% identity within the first 330 amino acids. The C-terminal regions are mostly unrelated; the α subunit contains 60 amino acids of unrelated sequence and the α' contain approximately 20 amino acids of unrelated sequence (Penner *et al.,* 1997). The α subunits can function in

the absence of the ß subunits *in vivo*, but with reduced activity. Although it is widely accepted to be a tetrameric enzyme, it has been reported that the α subunits of the enzyme in the nucleus of *Chironomus tentans* remain largely separate from the β subunits, and in fact the α subunits seem to be bound to other nuclear components (Stigare *et al.,* 1993). The ß subunits cause the monomeric α subunits to enter into a tetrameric conformation which might enable the two α subunits to phosphorylate each other, thus self-stimulate their activity (Tuazon and Traugh, 1991). The ß subunits usually act as protectors against proteolysis and heat denaturation; in addition, when bound to the α subunit pair they can change the enzyme's specificity to substrates and inhibitors (reviewed by Allende and Allende, 1995). The α subunits come together via the bridge forming homodimeric β subunits, to form the heterotetramer (~130kDa). The β subunit has been studied quite extensively by many groups in order to determine how its controlling effect is produced. Both its interaction with the α subunit and other substrates, as well as its association with itself have been studied. The β-β homodimer comes together via a homodimerization domain which is thought to be located between residues 20 and 145 (Kusk *et al.,* 1995). It seems that the C-terminal domain of the β subunit (last 45 amino acids) is essential for its interaction with the α subunit and may also be important for its positive effects on stability and activity (Chen and Copper, 1997). The N-terminal domain, which contains an acidic group of amino acids, seems to be important for its inhibitory effects. (Kusk *et al.*, 1995). An example of the β subunit's control of activity is the interaction of CK2 with the ribosomal protein L5 mediated via the β subunits (Kim *et al.,* 1996). The β subunit can also prevent phosphorylation of certain substrates, an example being calmodulin (Marin *et al.*, 1995).

The structure of the CK2 protein tells us much about its possible origins and of course its possible function. The amino acid sequence is highly conserved in different species, from *Drosophila* to human, especially in the catalytic domains of both the α subunits; the ß subunits are also highly conserved (see figure 2 and 4 for alignments). The motif 46 G R G K Y S E V 54 in the α subunit is important because it encompasses part of the binding site for ATP, and the presence of the tyrosine next to serine also appears in the cyclin-dependent kinases (cdk) family which suggests a possible common ancestor (Rihis *et al.,* 1991). The motif 74 K K K K I K R 80 represents a very basic stretch which could be involved in the binding to acidic regions of both substrates and inhibitors, such as those present in heparin, but also it has been postulated to be part of the nuclear localization signal for transport into the nucleus (Rihis *et al.,* 1991). The amino acid sequence of the CK2ß subunit shows similarities to the cyclin family, which have similar functions in that they both up- regulate the activity of their catalytic subunit and affect its substrate specificity (Ludeman *et al.,* 1993). They also seem to share an α-destruction box sequence which could mean that the ß subunit, like cyclin, might be targeted for ubiquitin-mediated proteolysis which gives some insight to its possible regulation during the cell cycle.

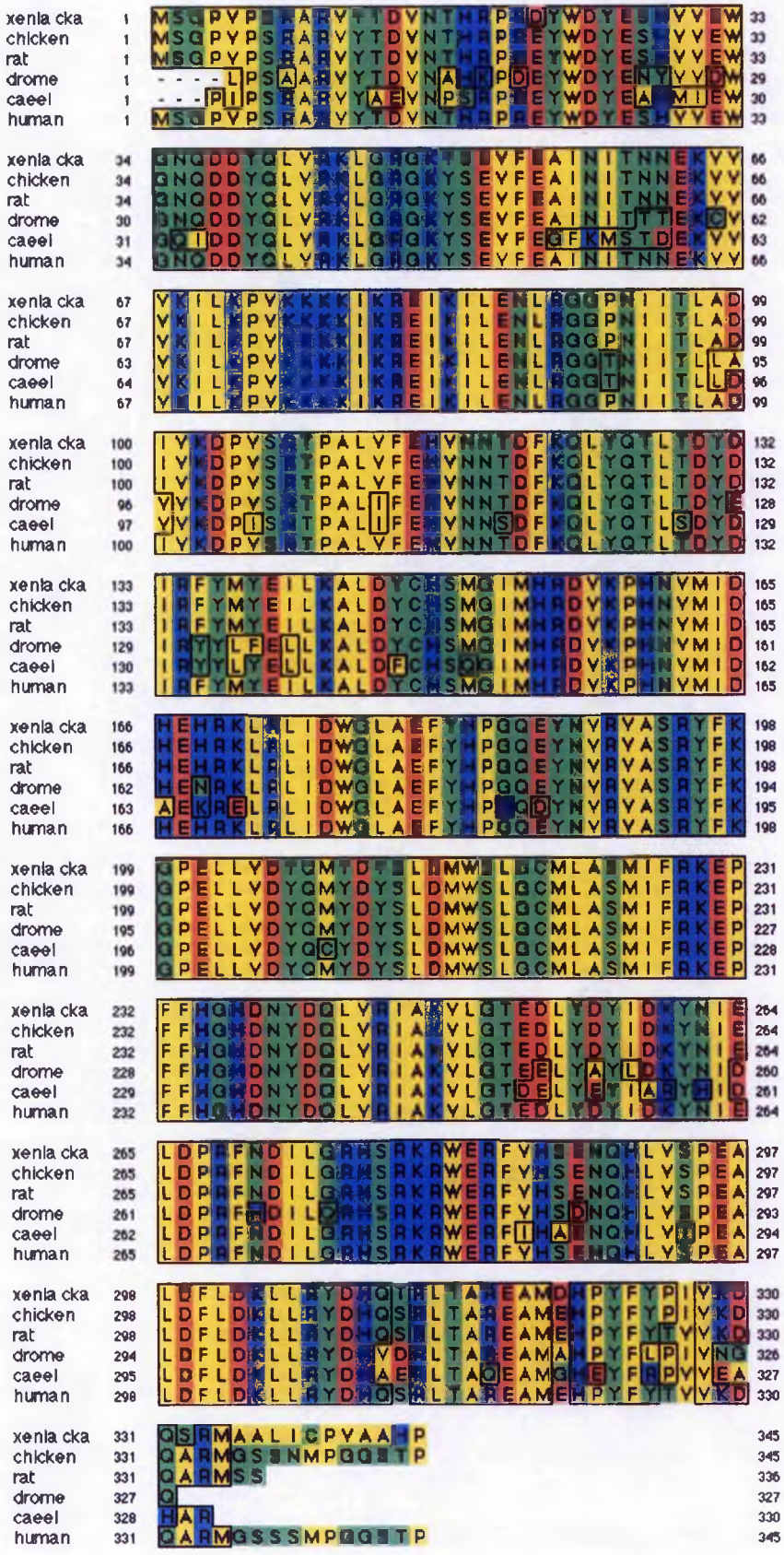
xenla cka
chicken
rat
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**Figure 2: CK2α Amino Acid Alignment:** Sequence alignment from different species. Red indicates acidic residues, blue indicates basic and yellow indicates neutral residues. Conserved regions are boxed. All sequences were obtained from EMBL data bank, aligned using GCG and presented in SeqVu.

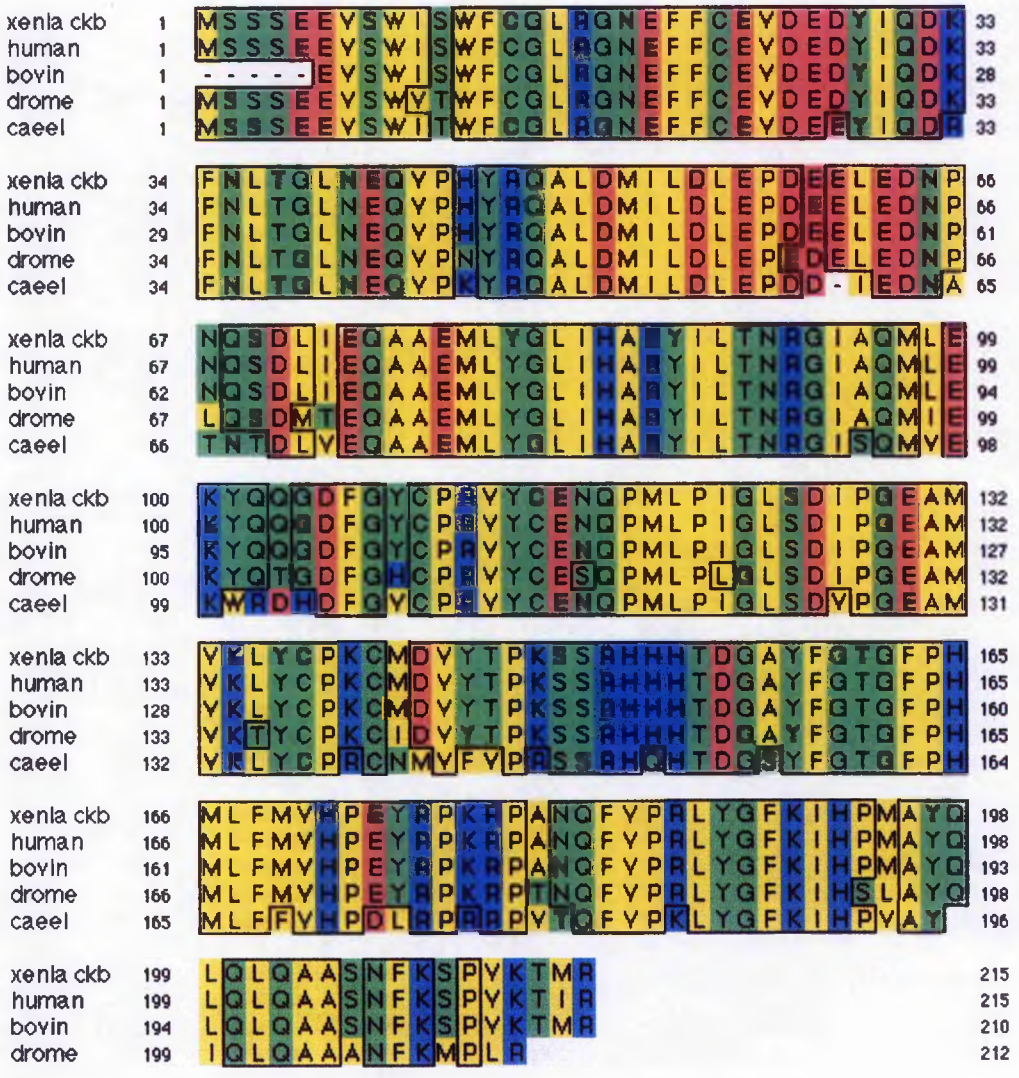



**Figure 3: CK2β Amino Acid Sequence Alignment:** Sequence alignment from different species. Red indicates acidic residues, blue indicates basic and yellow indicates neutral residues. Conserved regions are boxed. All sequences were obtained from EMBL data bank, aligned using GCG and presented in SeqVu.

The crystal structure of the α-subunit in *Zea mays* was recently obtained by Niefind *et al.* in 1998. The protein was shown to have a similar active site to cAMP-dependent protein kinase (cAPK) and cyclin-dependent kinase 2 (CDK2) bound to cyclin A; thus specific structural functionality was extrapolated from these known structures. The basic structure of the α-subunit consist of a β-strand rich N-terminal domain and an α-helical rich C-terminal domain between which lies the active site, as shown in figure 4. Helix αC, which lies in the β-strand rich N-terminal domain, is critical for substrate recognition (Pinna and Ruzzene, 1996) and is aligned towards the active site. This interaction may contribute to the highly active nature of CK2. This αC region contains a highly basic cluster of residues (74-KKKKIKREIK-83) which may interact with the β-subunit of CK2 (Sarno *et al.*, 1996). The β8 and β9 loop, which composes part of the activation site, could play an important role in catalysis due to the fact that they contain the magnesium chelating residue at Asp175. It helps orientate the γ-phosphoryl group of ATP properly and also the hydrophobic pocket for binding of nucleotide bases in which Ile174 plays a major role (Niefind *et al.*, 1998). The C-terminal lobe contains the αD helix which seems to be important for substrate binding and for the anchoring of ATP, but it does not have the well known hydrogen-bonding anchor site for the ribose moiety of ATP (Niefind *et al.*, 1998). The fact that ATP and GTP could both be used as co-substrates was explained due to the fact that the binding pocket is not highly conserved for ribose and triphosphate fixation. Thus it does not provide the specific hydrogen binding pattern that exists in other kinases (cAPK) that use only ATP or GTP. When ATP was bound to the purine binding site it bound in an disordered fashion, with the adenine base attached to a novel binding site (isoleucine instead of alanine) and with a low specificity (Niefind *et al.*, 1998).

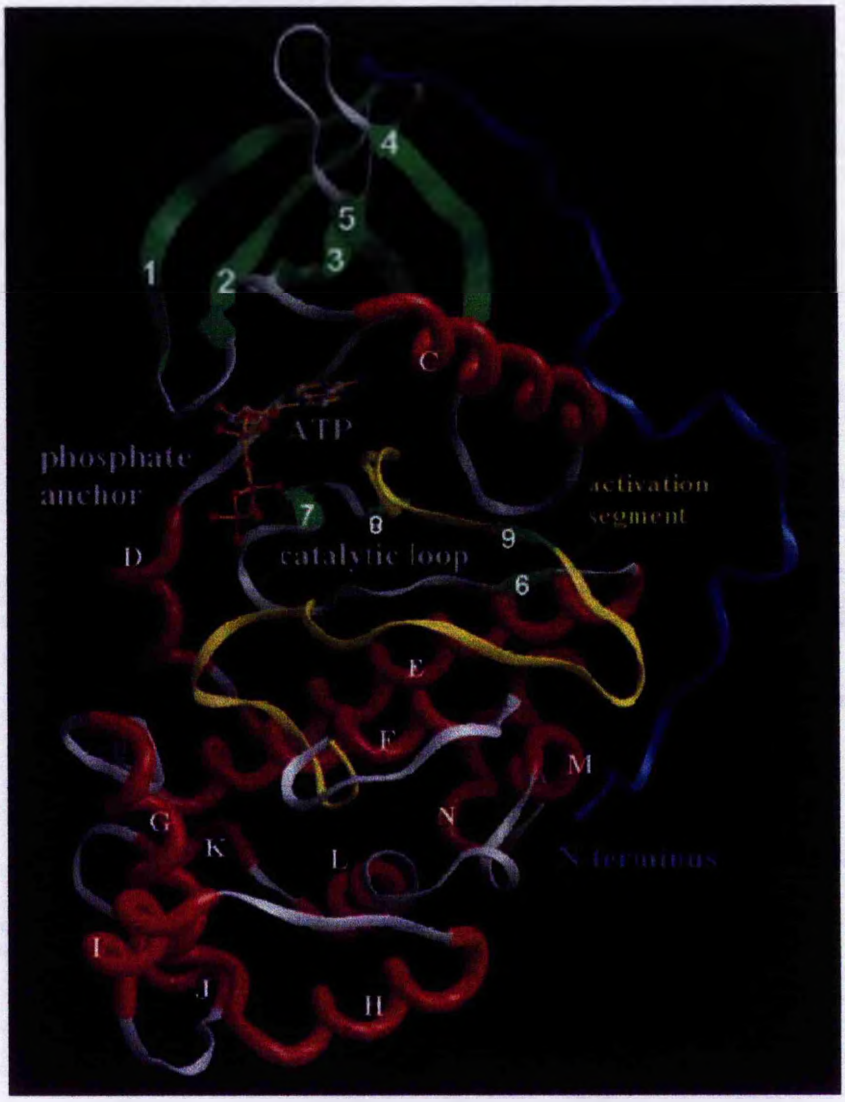



**Figure 4: Structure of CK2α**: Pictorial representation of CK2α subunit showing the N-terminal region in blue, activation region in yellow, the active site marked by a bound ATP molecule, and the α-helices (red) and β-sheets (green) are number and lettered. Taken from Niefind *et al.*, 1998.

*1.1.2 Function of CK2*: Specific functions for CK2 have been sought in many systems, but the general finding is that it is a multifunctional enzyme having many substrates. In *Saccharomyces cerevisiae* there have been at least four possible biological functions postulated: flocculation, cell cycle regulation, cell polarity, and ion homeostasis (reviewed by Glover, 1998). CK2 has also a plethora of substrates that it is known to phosphorylate. The diversity of these substrates makes it increasingly difficult

to define a definitive pathway to illustrate CK2's functions. For example, it can modify enzymes involved in nucleic acid synthesis (RNA polymerase I and II, DNA topoisomerase I-II, DNA ligase); transcription factors: nuclear oncogene products and tumor suppressor proteins (c-myc, large T antigen of SV-40, c-myb, N-myc, C-jun, retinoblastoma (RB)); signal transduction proteins ($p34^{cdc-2}$, protein kinase C, protein kinase A, insulin receptor, calmodulin, LD-lipoprotein receptor); protein synthesis factors (eIF3, eIF4B, eIF5, EF1ß, EF2ß, eIF2ß); cytoskeleton and structural proteins (ß-tubulin, myosin heavy and light chain, spectrin, tropin-T, Map-1B); other nuclear and nucleolar proteins (HMG 1, HMG 14, nucleolin, Np-B23, p120, pp35, p210, FK506) (reviewed by Allende and Allende, 1995). The (potential) importance of CK2 in regulating many cellular processes is illustrated by the wide range of substrates listed above. It will be important to explain how these different mechanisms may be linked together via this regulating enzyme.

Protein kinase CK2 is an important regulator of transcription and translation in both germ cells and somatic cell cycles. It has been deemed necessary for $G_0/G_1$ transition, early $G_1$, and $G_1$/S transition phases of the cell cycle (Pepperkok *et al.,* 1994). The effects of CK2 on transcription and translation events have been widely studied in many diverse mechanisms and cellular models. The phosphorylation of the Y-box proteins is thought to enhance their binding to mRNA (Murray *et al.*, 1991); it has also been postulated that Y-box phosphorylation could block the translation of mRNA (Braddock *et al.*, 1994). The association of CK2 with nuclear regulation factors, such as FGF-2 (Basic Fibroblast Growth Factor-2) has also been studied. FGF-2 was postulated to stimulate the activity of CK2 thus in turn activating potential transcription factors such as nucleolin (Bouche *et al.,* 1994). Nucleolin is a nucleolar phosphoprotein which is essential in the modulation of rRNA synthesis and pre-ribosome assembly. It is thought that nucleolin can bind to the non-transcribed spacer region of rDNA and it may be a

possible transcription site for RNA polymerase I (Bouche *et al.,* 1994). Nucleolin has been described to be an inhibitor of transcription *in vitro* when unphosphorylated, and when phosphorylated it supposedly does not interfere with transcription; therefore phosphorylation of nucleolin seems to be required for cell growth.

CK2 is found in greater amounts and with increased activity in cells that are actively proliferating, such as embryonic and tumor cells (Issinger *et al.*, 1993). It has been proposed that cdc-2 kinase (MPF) phosphorylates CK2 in order to up-regulate its activity. Progesterone-induced oocyte maturation seems to have only modest effects on stimulating CK2 activity; a 1.7 fold increase has been observed in *Xenopus laevis* oocytes (Cicirelli *et al.,* 1988). Although larger levels of increased cdc-2 kinases activity are seen after progesterone-induced maturation, it is possible that this kinase could activate CK2 activity at a later stage in embryonic development. It has also been shown that the activity of CK2 is enhanced by mitogenic factors, such as serum, insulin, IGF-I and EGF (Allende and Allende, 1995). This up-regulation could be due to post-transcriptional modification, and not to increased protein production. However, these findings have been the subject of some controversy and the exact effect of mitogenic factors in regulating CK2 is still unclear. Insulin is seen to induce phosphorylation of nucleolin in 3T3-442A cells. It is proposed that CK2 may play a role in the efflux of RNA that is observed in cell nuclei after the addition of insulin. The dose dependence of insulin-induced phosphorylation of nucleolin and the insulin-induced efflux of RNA are almost identical (Csermely *et al.,* 1992). This suggests that insulin-induced phosphorylation and dephosphorylation of nucleolin may regulate RNA transport through the nuclear membrane.

Whether CK2 directly participates in a signal transduction pathway, when regulated by either FGF-2 or insulin to phosphorylate nucleolin or if its activation is a general response to general growth factors, is unclear (Bouche *et al.,* 1994). It is still not

fully understood whether CK2 is unequivocally involved in intermediate steps of a signal transduction pathways, as is for example cAMP-dependent protein kinase, or in cascade events, as is protein kinase $R_2C_2$ (Sommercorn *et al.*, 1978).

Examinations of CK2 activators and suppressors make it even more difficult to assign a specific role, since these seem to be diverse and do not point to any single physiological mechanism. Known activators of the CK2 enzyme are the polyamines such as spermine and spermidine which increase the activity by 2-3 fold (Meggio *et al.*, 1992). Polyanionic compounds, such as heparin, are well known potent inhibitors (Hathaway *et al.,* 1980; Taylor *et al.,* 1987). This could be due to the fact that heparin is a high acidic compound and binds very tightly to CK2's catalytic domain which binds to acidic residues in its substrates (aspartic and glutamic acids), thus acting as a competitive inhibitor.

Other compounds such as quercetin (Cochet *et al.,* 1981) and 5,6-dichloro-1β-D-ribofuranosylbenzimidazole (DRB) are also well know inhibitors of CK2, but their exact mechanisms of inhibition is unknown (Zadomeni and Weinmann, 1984). Kinetic studies have shown that quercetin behaves as a competitive inhibitor towards the substrate and exhibits a high affinity for the ATP and GTP binding sites of other similar kinases (cAMP-independent casein kinase G type (CKG)) (Cochet *et al.,* 1981). DRB is a known inhibitor of RNA polymerase II transcription and it is thought that this is due to its inhibitory action on protein kinase CK2 (Zadomeni and Weinmann, 1984). Also it has been shown that DRB some how actually alters the chromosomal distribution of CK2-α through a time-dependent clearance after the addition of DRB, but again it is unclear what the mechanism of this clearance could be (Egyhazi *et al.,* 1998).

Nucleic acids such as, single stranded DNA, poly U and tRNA have also been shown to have an inhibitory effect on CK2, which may suggest a nuclear regulatory mechanism yet unknown (Gatica *et al.*, 1989).

CK2 is an example of a protein kinase that can use GTP as well as ATP as a phosphate source. It is also known for its inability to use $Mn^{2+}$ instead of $Mg^{2+}$ to support activity (Mulner *et al.,* 1988). These criteria are widely used in the identification of the CK2 enzyme activity since its cofactors, activators and inhibitors are for the most part unique for this enzyme.

*1.1.3 Role in nuclear import:* Protein kinase CK2 is a multi-functional enzyme and although most of this chapter has been focused on its nuclear activity there are other important roles which should be mentioned. There are many proteins that are transported to the nucleus via a nuclear localization signal (NLS). These proteins include the transcription factors, structural proteins and enzymes; they are needed faster, and in more concentrated amounts than simple diffusion can supply. They are also needed at different stages of the cell cycle, so a system of regulation is needed that can responded to external stimuli, cell-cycle progression and developmental cues. These specific proteins are selectively transported through the nuclear pore complex (NPC) via recognition of internal signals. Uptake of many of these proteins may be regulated by phosphorylation and dephosphorylation, particularly during embryogenesis. Protein kinases, such as protein kinase CK2 and $p34^{cdc2}$ kinase, have been shown to play important roles in phosphorylating sites in transported proteins, which is linked to accumulation of these proteins in the nucleus (Vancurova *et al.,* 1995).

There exists a barrier known as the nuclear envelope that separates the nuclear matrix from the cytoplasm. This barrier is a selectively permeable membrane, which contains many NPC, and allows only certain cellular components entrance into the

nucleus. Many proteins and nucleic acids shuttle constantly to and from the nucleus via these gates. Nucleic acids such as mRNAs, tRNAs, and rRNAs are the main exports while proteins seem to be the main imports(reviewed by Nigg, 1997). The NPC provides the channel for transport through the nuclear membrane and this complex measures about 9 nm in diameter (Davis, 1995). This allows diffusion of smaller molecules such as metabolites, ions and small proteins (40-60K) but provides an energy-dependent selective channel for larger proteins. The NPC is made up of over 100 proteins collectively known as nucleoporins, which form a complex of about 125,000kDa (reviewed by Nigg, 1997). Of the many hundreds of proteins that make up the NPC only a minority have been widely studied. Some of the more important ones which have been studied extensively have roles in nuclear docking and translocation. Proteins that have been identified for translocation are Ran-GTP/GDP cycle and nuclear transport factor-2 (NTF2) (Moore *et al.,* 1993). This is an energy dependent step in which docked proteins are shuttled through the pore complex via binding to Ran; this process is regulated by Ran GAP1 and RCC1. There are other proteins such as importin-α and β that are important in recognition and docking to the NPC (fig 5). Importin-α binds to proteins via a nuclear localization signal (NLS) and importin-β mediates docking to the NPC (Gorlich, *et al.*, 1995). There are two possible systems for the regulation of nuclear transport. The first is cytoplasmic anchoring and release. Proteins are bound or anchored in the cytoplasm and in response to a signal, such as hormones, are released by phosphorylation or by selective proteolysis and migrate to the nucleus. The second known regulatory pathway is by masking and unmasking of the NLS (Nigg, 1990). The nuclear localization signal contains a specific motif within the amino acid sequence. The NLS usually consists of basic residues in either one or two clusters and therefore are called either mono or bipartite NLS (Dingwall and Laskey,

1991). It has been well established that phosphorylation of the NLS in proteins effects the efficiency of transport of those proteins into the nucleus. It is thought that phosphorylation can activate the NLS by the addition of phosphates to residues near or within the NLS, which either change the protein conformation thus exposing the NLS, or by enhancing the binding of transport proteins (Karin and Hunter, 1995). This means that kinases and phosphatases must work as opposing forces controlling which proteins and when nuclear transport will occur. It is thought that some protein phosphatases and kinases are compartmentalized to restrict subcellular location and improve specificity. It seems that the targeting of proteins are controlled by a reversible phosphorylation. A balance between kinases and phosphatases controls phosphorylation and these enzymes appear to be placed in specific cellular areas to augment this control (reviewed by Faux and Scott, 1996). Accessibility to the substrates seems to play a major part in regulating the selectivity of these enzymes. Many kinases (PKA, PKC, and CK2) and phosphatases (PP-1, PP-2A and PP-2B) are know to participate in this nuclear transport regulation (reviewed by Faux and Scott, 1996).

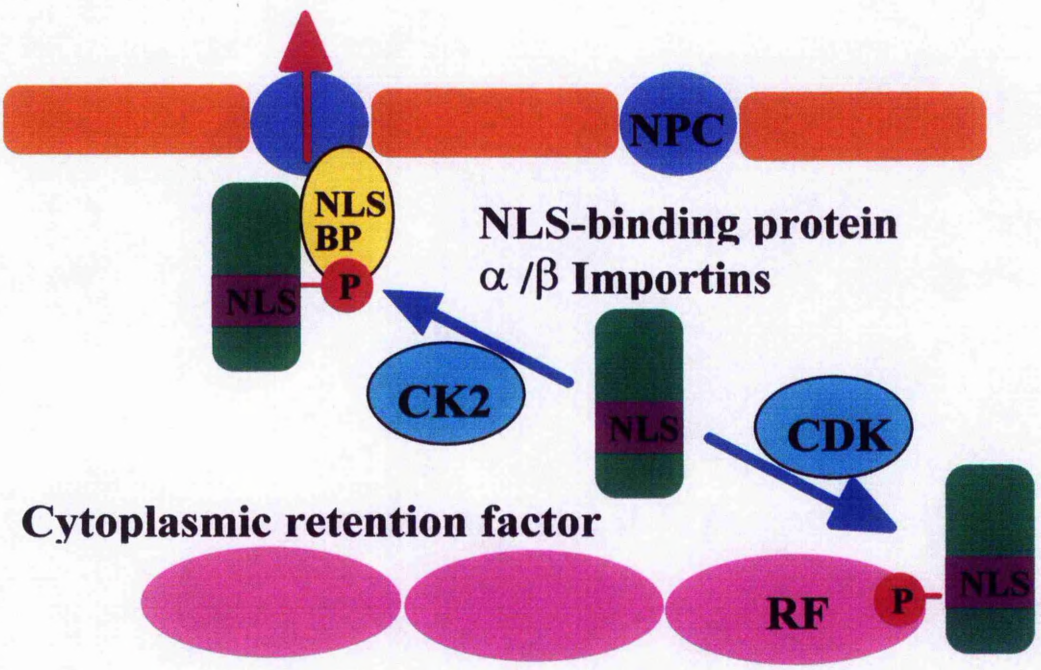



**Figure 5: NLS Function Regulated by Phosphorylation**: A possible role for CK2 in nuclear import. Phosphorylation of certain proteins affect the rate of nuclear import.

The SV40 T-antigen was one of the first proteins to be described having a nuclear localization single motif (PKKKRKV)(Kalderon *et al.*, 1984) and whose transport was enhanced by phosphorylation of a serine or threonine, between 10-30 amino acids away from the NLS, by the kinase CK2 (Rihs and Peters, 1989). It was shown that residues at 111 or 112 (both serine sites) enhance the transport of this protein after phosphorylation with CK2 (Rihs *et al.*, 1991).

The nuclear form of CK2 demonstrates an ability to modulate the activity of nucleolin; however cytoplasmic CK2 has also been shown to have a high binding and phosphorylation activity towards nucleolin (Dongxia *et al.*, 1996). Nucleolin has been shown to shuttle between the cytoplasm and nucleus, which raises the possibilities that CK2 might have some direct effect on this movement and movement of other related proteins into the nucleus (Borer *et al.*, 1989). Recently it has been shown that the bipartite NLS of nucleolin is phosphorylated by both CK2 and cdc2 kinase. Cytoplasmic

injection experiments, into *Xenopus* oocytes, demonstrated that the presence of both these sites leads to increased nuclear import and that the phosphorylation of the CK2 site further increased the rate (Schwab and Dreyer, 1997). The cdc2 sites had a dual role in controlling import. The presence of the cdc2 sites, within the nucleolin sequence, increased nuclear import, whereas the phosphorylation of these sites promoted cytoplasmic localization (Schwab and Dreyer, 1997). This combination of CK2 enhanced import and cdc2 induced cytoplasmic anchoring provides nucleolin with a powerful cell-cycle dependent regulatory mechanism.

Nucleoplasmin, a large pentameric nuclear protein with distinct head and tail domains, has been extensively studied, and it has been found that the rate of its nuclear transport is proportional to the degree of phosphorylation by CK2. For example, nucleoplasmin was treated with alkaline phosphatase, which removes 70% of the phosphates from the protein, and was labeled with $^{35}S$. It was then microinjected into the cytoplasm of *Xenopus* oocytes (Vancurova *et al.*, 1995). The observations were that treated protein accumulated in the nucleus much slower then the untreated protein. It was also observed that CK2 co-purified with nucleoplasmin, which suggests that not only does CK2 facilitate transport, but it is also transported with nucleoplasmin for possible use in the nucleus (Vancurova *et al.,* 1995). In summary, several studies have emphasized the importance of protein kinases and phosphatases as regulators of protein transport.

**1.2 Oocyte Development**

*1.2.1 The use of Xenopus oocytes*: CK2 activity has been previously investigated in relation to oocyte development in the South African clawed toad (*Xenopus laevis*). Oogenesis in *Xenopus* is a suitable model for experimentation due to the fact that there is an enormous wealth of developmental information already collected, and due to the large

size and relative ease of collecting its oocytes. Work was first done on *Xenopus* oocytes in the late 50s and early 60s when they were used to study nuclear transplantation (Fischberg *et al.,* 1958; Gurdon, 1960). Work continued, using these oocytes, because biochemical experiments, especially dealing with extraction of nucleic acids, was found to be much simpler and more reliable than those using oocytes from other species such as *Rana pipiens*. *Xenopus* oocytes have also proven to be an invaluable model for studying transcription and translation, nuclear import, protein secretion, protein compartmentalization, protein modification and many other aspects of cell biology.

*1.2.2 Classification of oocytes*: *Xenopus* oocytes grow asynchronously, which makes collection of any and every stage of oocyte very convenient. They are classified into six major stages according to size and pigmentation (Dumont, 1972). The smallest oocyte is classified as stage I (up to 100μm) and the largest stage VI (1200μm; see fig. 6). The time taken to develop from the smallest stage to the largest is several months. Oocyte development can also be stimulated using hormonal induction, usually follicle-stimulating hormone (Wallace, 1985). When stage VI is reached development is arrested at late prophase I of meiosis (diplotene), but such oocytes can be induced to mature in vitro by adding progesterone (Taylor and Smith, 1986).

**Stages: I II III IV V VI**

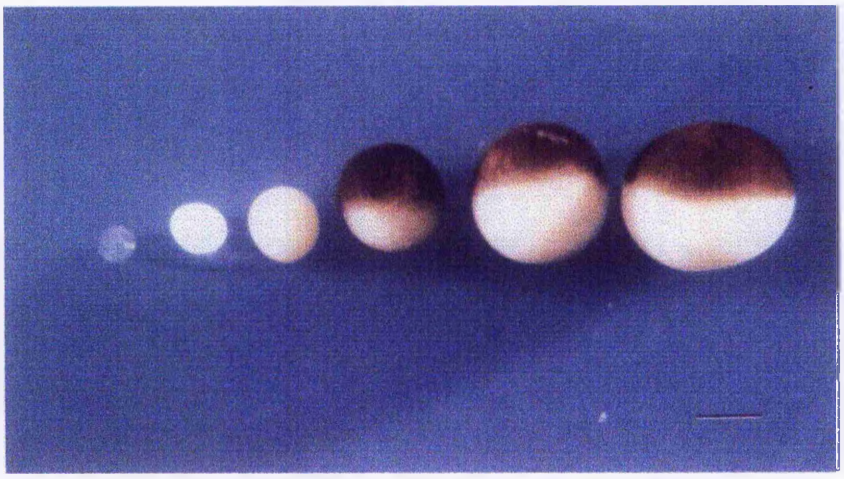


**Figure 6: Xenopus oocyte stages**: Stage I oocytes are 50-100μm in diameter and are translucent. Stage II oocytes are ~ 450μm and are opaque white, vitellogenesis (the deposition of yolk) starts at this stage. Stage III oocytes are 450-600μm and show the beginning of pigmentation deposit. Stage IV oocytes are 600-1000μm and have differentiated animal and vegetal hemispheres. Stage V oocytes are 1000-1200μm and show clear delineation of hemispheres: the animal hemisphere appears light brown. The final stage VI oocytes are 1200-1300μm have a white band separating the two hemispheres and are post-vitellogenic and are ready for maturation. Bar represents 500 μm. Taken from Kay and Peng 1991.

*1.2.3 Developing oocyte*: During oogenesis the changes in gene expression and rate of protein synthesis increase dramatically, especially with the onset of maturation and after fertilization. The accumulation of yolk proteins starts at stage II (vitellogenesis) and continues until early stage VI. Large stores of ribosomes ($10^{12}$) and mRNA molecules ($2x10^{11}$) accumulate per oocytes over this period of oogenesis (Davidson, 1986). The amount of RNA of all types, increases from about 0.04 μg in stage I to over 4 μg per oocyte in stage VI. Of this RNA only 1% is messenger RNA, which encodes for over 20,000 distinct protein sequences. The rest is mostly ribosomal (90%) and transfer RNA (4%) (Davidson, 1986). At stage VI of oogenesis, growth is

blocked at the diplotene stage of development around the first meiotic division in prophase I. Progesterone then activates the maturation process and produces a cascade of events. Oocytes complete the first meiotic division and continue through meiosis II to metaphase, in which they arrest again as unfertilized eggs. During maturation the nuclear envelope breaks down (germinal vesicle breakdown, GVB), exposing the chromosomes, which have condensed from their active state (lampbrush chromosomes), to cytoplasmic factors. These events enable the first meiotic division to take place enabling the G2 to M transition of the cell cycle. The cytoplasmic factors that make up this machinery are stimulated by hormones and are known as the maturation-promoting factor (MPF). They are thought to consist of the enzymes cdc2 kinase and cyclin B (Sunkara *et al.,* 1979; Colas and Guerrier, 1995). During MPF activation in *Xenopus,* a burst of phosphorylation is observed which is independent of cyclic-AMP-dependent protein kinase, a known stimulator of MPF (Mulner-Lorillon *et al.,* 1988). As mentioned before, CK2 has been implicated in cell division elsewhere and there is a phosphorylation increase during meiotic cell division in *Xenopus* oocytes. Earlier work has also shown that CK2 is a target for cdc2 activation during MPF induced phosphorylation cascade (Belle *et al.,* 1990). These findings made it imperative to study the activity of CK2 in this system to determine its exact influence during this process.

*1.2.4 CK2 activity during development*: There have been limited studies on the activities of CK2 in *Xenopus* oocytes by many different groups which serve to highlight the importance of this enzyme in the development of oocytes. The enzyme was first purified and characterized from *Xenopus* ovary in 1988 (Mulner-Lorillon *et al.,* 1988). It was referred to as a casein kinase II-like enzyme since its exact sequence was unknown. The purified enzyme was injected into full grown oocytes and its biological effects were investigated. Injection of the enzyme was shown to increase the activity of the MPF,

thus augmenting meiotic maturation, but it seemed to inhibit meiotic cell division by blocking the effects of progesterone (Mulner-Lorillon *et al.,* 1988).

The sequences of the α and β subunits were first determined for *Xenopus* in 1992 using a probe derived from the human and *Drosophila* sequences (Jedlicki *et al.*, 1992). The results showed a very high sequence conservation between human and *Xenopus,* with the α subunits showing a very high conservation at the amino end: about 80% identity in the first 323 amino acids, and the β subunits showed high conservation with only 2 amino acids difference between *Xenopus* and human (Jedlicki *et al.*, 1992). Estimates have been made of the amount of CK2 mRNA and enzyme present at different stages of oogenesis (II-VI) and it was found that the amount of message actually increases 2 to 3 fold from stage II to stage VI for both the α and β subunit. There are approximately 5 x $10^7$ molecules of CK2α mRNA and 1 x$10^7$ of CK2β mRNA in full grown oocytes (Wilhelm *et al.,* 1995). The noted increases are interesting, since it has been claimed that peak levels of most stored (maternal) mRNA are reached in oocytes at early stages (I-II) (Golden *et al.,* 1980; Davidson, 1986). The graded increase in mRNA encoding CK2 throughout oogenesis is puzzling. Another observation is an increase in enzymatic activity of about 12-15 fold from stage II to stage V, which means that CK2 is being used throughout oogenesis and that its stores must be constantly replaced; however the exact purpose of CK2 during these stages of development is unknown (Wilhelm *et al.*, 1995).

The β subunit has been shown to have an effect on oocyte maturation that is independent from the α subunit. It has been shown that the β subunit binds to Mos and inhibits its ability to stimulate Mos-mediated mitogen-activated protein kinase (MAPK). Mos is necessary and sufficient to initiate oocyte maturation, and when CK2β is bound, this inhibits progesterone induced maturation. Also, when the levels of CK2β enzyme

are decreased, MAPK activity is increased when treated with low concentration of progesterone (Chen and Cooper, 1997). The activity of Mos, MAPK, MMK and MPF seem all to be linked, and dependent on phosphorylation activation. Without it the oocyte cannot commit to germinal vesicle breakdown, chromosome condensation, suppression of DNA replication, entry into meiosis II, and metaphase arrest (Chen and Cooper, 1997). It seems that CK2β may possibly set a threshold level for Mos activity to activate oocyte maturation.

### 1.3 Phosphorylation of Proteins in Maternal mRNP Particles

*1.3.1 mRNP phosphoproteins*: During oogenesis, in *Xenopus laevis*, the oocytes increase many times in size and accumulate cellular components required for early embryonic development. Cellular components such as mRNA, tRNA, histones and ribosomes are synthesized and packaged for use during the rapid cell divisions of early embryogenesis. A pool of about $2x10^{11}$ molecules of mRNA/oocyte is established by vitellogenesis and is mostly maintained until oocyte maturation. This maternal mRNA is the source of new protein through maturation, fertilization and embryonic cleavage stages. At mid-blastula, zygotic gene expression is initiated and the maternal information is replaced (Davidson, 1986).

In early oogenesis (and in spermatogenesis) mRNA is stored as mRNP by the binding of specific proteins to the mRNA. This occurs in order to prevent mRNA from premature translation and possibly might also protect it from degradation. The mass ratio of protein to RNA is about 4 to 1 in ribosome-free mRNA (Darnbrough and Ford, 1981; Cummings and Sommerville, 1988). Some of these bound proteins could be also necessary for the translation of the mRNA later in early embryogenesis, but this has not been proven. Protein kinase CK2 activity may be required for the phosphorylation of some of these proteins in order to help assemble or to stabilize the mRNP particle.

There are four main proteins associated with the mRNA which have the apparent masses on SDS-PAGE of 60, 56, 54, and 50 kDa (Darnbrough and Ford, 1981; Cummings and Sommerville, 1988). In addition, a kinase with the properties of CK2 co-sediments with the mRNP particles. The two proteins pp60 and 56 kDa are phosphorylated *in vivo* during the developmental stages in which mRNA is repressed (Cummings *et al.*, 1988). These proteins have been identified as FRGY-2a and FRGY-2b, and are members of the Y-box family of proteins (Tafuri and Wolffe, 1990). The name Y-box refers to a recognition sequence, containing an inverted CCAAT-box, which is found in the promoter region of MHC class II genes to which these proteins are known to bind (Didier *et al.*, 1988). Y-box proteins are regulators of transcription and translation and have been shown to bind to mRNA in order to prevent translation; this process is known as "masking" (reviewed in Sommerville and Ladomery, 1996a). The primary structure of FRGY2a (pp60) and FRGY2b (pp56) became apparent with the cloning of their cDNA (Tafuri and Wolffe, 1992; Murray *et al.*, 1992). The two sequences are very similar, indicating that FRGY2a and 2b are encoded by pseudoalleles (fig. 44). This situation is not unusual because *Xenopus laevis* is believed to have evolved as a tetraploid species. FRGY2a/b consist of a cold-shock domain and alternating regions of basic and acidic amino acids. The cold shock domain (CSD) is made of five antiparallel β-strands forming a β-barrel structure. This structure binds single stranded RNA and DNA very efficiently (Schindelin *et al.*, 1994; Newkirk *et al.*, 1994). This binding of RNA and DNA via the CSD suggests that the Y-box proteins are potential regulators of transcription and translation. The CSD actually proved essential for the binding of FRGY2 to Y-box containing promoters in *Xenopus* DNA (Tafuri and Wolffe, 1992). The regulation of the binding of Y-box proteins to nucleic acids is one of the fundamental questions that still remains. Examination of the Y-box proteins and the CSD revealed that the CSD is associated with a series of alternating basic and acidic

charged islands. These alternating charged islands are collectively known as the "tail domain". The acidic regions contain potential CK2 phosphorylation sites and these are thought to promote protein multimerization and to stabilize RNA-binding (Tafuri and Wolffe, 1992). It has been demonstrated that phosphorylation is necessary for mRNA masking, but its exact purpose is still unknown (Kick *et al.*, 1987; Cummings and Sommerville, 1988; Murray *et al.*, 1991; Murray, 1994). Early reports suggest that these proteins are phosphorylated at serine residues specifically at position 149 in FRGY-2a and b and at position 125 only in FRGY-2a (Deschamps *et al.*, 1997) but what purpose this phosphorylation serves is unclear. The phosphorylation studies claim that phosphorylating FRGY-2a and 2b does not effect their ability to bind to RNA, and it is suggested that their phosphorylation state does not influence the initial masking of mRNA in oocytes (Deschamps *et al.*, 1997). Other possibilities are: that phosphorylation controls the interaction of FRGY2 molecules to form dimers or multimers on the DNA or mRNA with other proteins; and the fate of the assembled mRNPs. It has been shown that microinjection of inhibitors of protein kinase into mid-oogenic oocytes has increased the rate of endogenous protein synthesis by a factor of two to three. The increased activity seems to be due to mobilization of mRNA into polysomes instead of increased translation efficiency (Sommerville, 1990). Injection experiments which introduce reporter RNA and antibodies against CK2 into oocytes resulted in about 20% of the potentially translatable RNA being released from masking, which points to the importance of CK2 activity in the masking process. Similar results were obtained when oocytes were treated with kinase inhibitors such as DRB or quercetin, but treatment with rutin (inactive form of quercetin) had no effect (Braddock *et al.*, 1994). Progesterone-mediated unmasking of mRNPs, which is needed in promoting the maturation of oocytes, has been found to be blocked by phosphatase inhibitors, such as okadaic acid, which suggests the phosphorylation is indeed necessary

to maintain masking, but phosphorylation of which proteins by what kinase is unclear (Braddock *et al.,* 1994). These results do point to the importance of CK2 in the control of translation of mRNPs, and if correct, the levels of phosphorylation of mRNP particles should coincide with the presence of the CK2 activity and with the non-translating state of message. Of course the converse should also be true: that during translation of message the associated CK2 level should be low as well as the level of phosphorylation of mRNP.

| Protein 1 | Protein 2 | References |
|---|---|---|
| mRNP4 | mRNP3 | Darnborough and Ford, 1981<br>Murray *et al.*, 1994 |
| pp60 | pp56 | Kick *et al.*, 1987<br>Crawford and Richter, 1987 |
| p56 | p54 | Murray *et al.*, 1991 |
| FRGY2a | FRGY2b | Tafuri and Wolffe, 1990 |

**Figure 7: Variations in naming of major mRNA masking proteins in *Xenopus* oocytes**: The four major variations in nomenclature of FRGY2a and FRGY2b.

*1.3.2 Expression of CK2 during oogenesis*: The levels of CK2 message would be expected to be expressed at different levels during different stages of development, depending on whether maternal mRNA should be stored or translated. However CK2 has many other functions besides the masking of mRNA, and since its mRNA may itself be under translational control, little can be concluded from the levels of message seen. The levels of mRNA have been calculated to be, in stage VI, $5x10^7$ mRNA per oocyte for the α subunit, and $1x10^7$ for the ß subunit (Wilhelm *et al.*, 1995). During oogenesis the relative levels of mRNA for CK2 increase 2.5 fold for the α subunit and three fold for the ß subunit from earlier stages (II) to later oocyte stages (V-VI) (Wilhelm *et al.*, 1995). The exact levels of CK2 mRNA is presently being disputed due to comparison with earlier work done on levels of other mRNAs, such as GTP-binding protein (100-

fold less), Ras mRNA (threefold less), and A-type and B-type cyclins which remain constant from stage two to stage VI in *Xenopus* oocytes (Wilhelm *et al.,* 1995). Why the levels of CK2 message appear high compared with other messages, and even increase through oogenesis while mRNAs for other important growth proteins remain constant, is unanswered.

It has been shown that CK2 is an abundant protein in the nucleoli, which leads to the speculation that CK2 might be required for the synthesis of ribosomal RNA (Wilhelm *et al.,* 1995). This would explain the increased levels of CK2 mRNA, since the synthesis of ribosomal RNA reaches maximum rates though mid to late oogenesis. After fertilization of the oocytes, the levels of CK2 dramatically decrease. The FRGY2a/b masking proteins are no longer phosphorylated, although they are still present in the mRNP (Sommerville, 1990). During early cleavage to gastrulation, the level of activity of mRNP-bound CK2 drops by a factor of seven. This could be due to increased levels of protein phosphatase activity and/or kinases inhibitors that are observed to accumulate at the end of oogenesis and into embryogenesis (Sommerville, 1990). These changes in phosphorylation seem to coincide with the formation of polysomes and translation of the maternal mRNA (Sommerville, 1990).

*1.3.3 Export of RNA from the nucleus*: Export of mRNP particles from the nucleus is another mystery of oocyte development. It is well known that after mRNA is transcribed it is exported to the cytoplasm for translation. During oocyte development mRNA accumulates and is stored in the cytoplasm for future use during fertilization. How mRNA is exported has been widely studied and it is thought that there must be a nuclear export signal (NES). It is thought that proteins that shuttle between the nucleus and cytoplasm act as carriers of RNA. It is on these proteins that the signal for export is thought to exists and not on the RNA itself. This signal has been discovered on proteins

such as Iκb, Rev from HIV and TFIIIA and is believed to be a leucine-rich peptide sequence. The yeast protein Np13p is a protein which shuttles back and forth between the nucleus and cytoplasm also binds to RNA. Mutation in the NES of this protein has shown a marked decrease in export of poly(A) RNA from the nucleus (reviewed by Lee and Silver, 1997). The contribution to mRNP export of structures within the mRNA itself is another aspect being studied. It is thought that certain modification to the mRNA such as a cap addition may facilitate its transport. Also the presence of introns may trap the mRNA in spliceosomes which would inhibit premature export of unspliced RNA. It is also a possibility that modifications of the bound proteins may facilitate export by enhancing their association with export factors like α/β importin, Ran or transportin (reviewed by Nigg, 1997). Such modifications could result from phosphorylation, dephosphorylation or protein cleavage. Exactly which proteins, and what modifications are necessary, continues to be investigated. It is also important to establish how these complexes interact with the nuclear pore complex, and to what external signals they respond.

## 1.4 Maternally Expressed Histone Deacetylase and Development

*1.4.1 Histone function and modification*: The *Xenopus laevis* clone AB21 (accession number X78454) was first recognized as a candidate homologue to the yeast gene regulator RPD3 (Vidal and Gabor, 1991). This was later shown to be a homologue of the human histone deacetylase HDAC1 (Taunton *et al.*, 1996). Histone deacetylase is a maternal enzyme expressed throughout oogenesis and into early embryogenesis in *Xenopus laevis*. Its function is to aid in the formation of tightly packed nucleosomes after new chromatin has been replicated. Nucleosomes are composed of core histones, one of them being pre-acetylated histone H4. Histone H4 is acetylated at lysine residues

(residues 5 and 12) by histone acetyltransferase HAT B (Sobel *et al.*, 1994). When H4 is acetylated it reduces it net positive charge, which has been shown to disrupt the tight binding of the nucleosome, and thus could allow the binding of transcription factors to promoters. The acetylation and deacetylation of core histones plays an important regulatory role in the transcription and replication of chromatin.

The interaction of several other protein factors with histone deacetylase results in the formation of complexes which function as a regulators of gene expression (reviewed by Wolffe 1996, 1997). HDAC1 has been shown to associate with proteins such as RbAp48, Sin 3, and N-Cor (Taunton *et al.*, 1996; Alland *et al.*, 1997; Heinzel *et al.*, 1997). These complexes are believed to function as repressors of gene transcription by targeting the deacetylase activity to specific chromatin sites.

*1.4.2 Expression of HDACm during oogenesis*

During *Xenopus* oocyte development, a maternal form of histone deacetylase (HDm; Ladomery *et al.*, 1997; HDACm Sommerville *et al.,* 1998) is expressed thoughout development. It steadily increases, along with non-chromosomal histone proteins as components required for the formation of new chromatin during the rapid cell cleavages leading to blastula. During maturation and early embryogenesis, transcription activity is not detected and maternal stores of histones and assembly factors produced new chromatin from the rapidly replicating DNA. It is during this assembly of newly formed chromatin that histone deacetylase removes the acetyl groups from the core histones, thus stabilizing the chromatin into nucleosomes. The expression of HDACm decreases after fertilization at around mid-blastula. This corresponds to when the cell-cycle slows down and cells begin differentiation (Sommerville *et al.,* 1998).

The regulation of activity and transport of HDACm is one major question still left unanswered. Within the HDACm amino acid sequence there are several potential

phosphorylation sites, specifically motifs recognized by CK2. This protein kinase has an expression pattern similar to that of HDACm (Wilhelm *et al.,* 1995) and, as discussed previously, has been shown to regulate the function and nuclear import of many substrates (reviewed by Allende and Allende, 1995). The phosphorylation state could be a regulatory mechanism controlling the association of histone deacetylase into protein complexes, controlling the substrate recognition specificity, level of activity, or nuclear import of newly synthesized HDACm.

## 1.5 Project Aims

Protein kinase CK2 activity has been shown to be an important part of the control of transcription, translation and intracellular transport. Its exact function and regulation has been postulated, but more evidence is needed. The work recorded here concerns the role of protein kinase CK2 in modifying histone deacetylase, specifically via the phosphorylation of HDACm, and in determining translation of masked messages, specifically via the phosphorylation of Y-box proteins. The project aims to measure the expression and activity of CK2 in developing oocytes and to study its effects on these specific substrate proteins which are also important for oocyte development. Before interaction of CK2 with HDACm were studied, CK2 expression was studied, in detail, to get a better understanding of its concentration and activity as oocytes develop. The nuclear import of HDACm was suspected to be affected by phosphorylation by CK2, consequently this aspect was studied in greater detail. The effect of CK2 on mRNP particles had been investigated previously, but further work was considered to be necessary in order to understand better the role of phosphorylation of Y-box proteins.

# Chapter 2: Methods

## 2.1 λZap cDNA Library Screening

*2.1.1 Library construction:* A cDNA library was prepared by Dr. Sommerville following the instructions in the λZAP manual. cDNAs were prepared from poly(A)$^+$ mRNAs extracted from polysomal mRNP from previtellogenic oocytes, the idea being to select for mRNAs encoding proteins required at a high levels during this phase of development.

*2.1.2 Plating bacteriophage λ*: The plating cells for λZAP transformation were a strain called XL1-Blue. Individual colonies of these plating cells were picked from a plate and used to inoculate a 5 ml Luria broth (LB, per litre: Bactotryptone 10g, Bacto-yeast extract 5g, and NaCl 10g) which was grown overnight at 37°C. Next 40 ml of fresh LB was inoculated with 1 ml of the overnight culture to which maltose and MgS04 were added to a final concentration of 0.2% (w/v) and 10 mM respectively. The cells were grown with vigorous shaking until they reached an $OD_{600}$ of about 0.5, after which they were spun at 3,000 g at 4°C for 5 minutes, and resuspended in 4 ml of ice-cold sterile 10 mM MgS04. 4ml of top agarose (0.8 % agarose in LB) was melted and placed in a 45°C water bath. 100 µl of plating cells were infected with an appropriate dilution of bacteriophage in SM buffer (per litre: NaCl 5.8g, $MgSO_4$ 2g, Tris base 6.05g, 2% gelatine 5ml and HCl to pH 7.5). Cells were infected for 15 minutes at 37°C, and then plated out by mixing them with 4 ml of top agarose cooled to 45°C and pouring the mixture onto pre-warmed plates. The top layer was allowed to set for 15 minutes on the bench and then the plates were placed inverted into a 37°C incubator. Plaques appeared after approximately 6 hours.

For plating out the library in the primary round of screening, larger plates were preferred (150 mm diameter) in order to plate out 10,000-20,000 pfu (plaque forming units) per plate.

*2.1.3 Expression of plaque fusion proteins*: Nitrocellulose filters were prepared by wetting them in a sterile solution of 10 mM IPTG and then dried before use. When the plaques were sufficiently developed, the filters were carefully overlaid on the plaques. The plates were placed back into the oven for a further 3-4 hours. This allowed the fusion proteins to be expressed. The filters and the plates were marked with a needle, covered in Indian ink, to enable orientation and identification of the plaques. The filters were lifted off the agar and labelled for screening. Afterwards, they were placed into TBS containing 2% filtered skimmed milk overnight. Meanwhile, the agar plates were stored at 4°C for later picking of positive plaques.

*2.1.4 Identification of positive plaques using antibody screening*: The nitrocellulose filters were probed with antibodies obtained either from an outside source (CKα chicken), or from a rabbit that had been injected with fusion protein (CKα). The procedure was the same as described in the antibody production section 2.14. The DAB reaction produce small brown dots that corresponded to the plaques that were making a protein which was recognised by the antibody. The filter was place on to a sheet of acetate. The outline of the filter and the position of the orientation marks were marked onto the acetate. Next, the position of the positive signals were marked and the acetate was aligned with the agar plates.

*2.1.5 Picking positive plaques*: Positive plaques were cored out using a 1 ml Gilson pipette fitted with a truncated tip and transferred to a 1.5 ml microcentrifuge

tube containing 500 µl of SM buffer. A drop of chloroform was added for storage at 4°C. Phage particles diffused out of the agar plug into the SM buffer; typically, a plug gave a titre of approximately $10^5$ pfu/ml. To purify positive plaques, the initial stocks were plated at lower densities until a single positive isolated plaque could be picked.

*2.1.6 Recovery of pBlueScript from λZAP:* 2 x $10^7$ XL-1 cells in 200 ml were combined with 200ml of the λZap phage (1 x $10^5$ phage) that were obtained from the plaque picks. Also 1 µl of R408 helper phage was added (1 x $10^3$ phage). They were incubated for 15 minutes in 37°C water bath. The mixture was then heated for 20 minutes in a 70°C water bath and spun at 5,000 rpm for 5 minutes. The supernatant was removed and this was stored at 4°C until needed. This phagemid stock was used to reinfect XL-1 cells, so that ampicillin resistant transformants were produced containing the sequence of cDNA. Only the XL-1 cells that were infected with the phagemid carrying pBlueScript would have ampicillin resistance. These were plated on agar plates containing ampicillin at 50 µg\ml.

*2.1.7 Recovery of plasmid DNA using Wizard Minipreps*: Single colonies of XL-1 were picked and grown overnight in 5 ml of LB. This culture was spun down and supernatant was removed. The DNA from these cells were extracted using the Wizard Plus SV Miniprep (Promega). The standard Wizard Miniprep protocol was used in obtaining purified plasmid DNA. The kit is a small scale purification of plasmid DNA, using spin column to wash bacterial lysate through a filter which captures the DNA and then is eluted from filter using nuclease free water.

*2.1.8 Analysis of DNA using agarose gel electrophoresis*: Samples of DNA were digested for 1-2 hours in small volumes (10-20 µl), containing the required restriction buffer and restriction enzyme. Removal of RNA was done by adding RNase to a final concentration of 50 µg/ml, ribonuclease A, and 50 units/ml of ribonuclease $T_1$ to the digests, in the final 20 minutes of their restriction. 5 µl of gel loading buffer (0.25 % Bromophenol blue, 0.25% Xylene cyanol, and 30% Glycerol in $dH_20$) was added to the restriction reaction before loading onto an agarose gel for electrophoresis. The concentration of agarose in the gels varied between 0.8 and 2%, depending on the size of DNA fragments being analysed: 0.8% agarose was best for separation of larger fragments (> 2 kb), whereas 2% agarose favoured the separation of smaller fragments (500 bp and less). The agarose was dissolved in 30 ml of TAE buffer (40mM Tris-acetate and 1mM EDTA) by melting in a water bath on a Bunsen burner. It was then placed into a water bath at 45°C for at least 30 minutes to bring the temperature down to near its setting. Next, ethidium bromide was added to a final concentration of 0.5 µg/ml before pouring the gel. The gel was covered in TAE buffer, and the restriction digests loaded (up to 15 µl per well). Samples were run at 40-100 V and the DNA bands were visualised using a 300 nm UV-light transilluminator.

*2.1.9 Purification of DNA from agarose*: Once the DNA was determined to be of the correct size it was often cut from the agarose gel, in order to recover and purify it. The band was cut using a sterile razor blade while being viewed on the transilluminator, on low power. This was to ensure that only the band representing the required DNA was excised. The DNA was removed from the agarose gel by

purifying it using the Nucleon™ Easiclene Kit: for the rapid isolation and purification of DNA (Scotlab). The method used was the standard protocol that was included with the purification kit by the manufacturer. The theory behind the kit is that the agarose is melted away in 6M NaI and the freed DNA is bound to a silica-based matrix (glassmilk). The matrix is washed with a "wash" buffer (50mM NaCl, 10mM Tris.HCl pH 7.5, 2.5 mM EDTA, 50% v/v ethanol), which removes the ethidium bromide and other impurities, and the purified DNA is then eluted in warm $dH_2O$. In general, 5 µl of glassmilk was added to samples containing 5 µg or less of DNA, and an extra 1 µl for every 0.5 µg of DNA above 5 µg. This purified DNA could be used for various other techniques, such as sequencing or ligation into expression vectors.

**2.2 DNA Sequencing**

Sequencing was carried out by the DNA automated sequencing unit located in the Irvine building. Samples must contain 0.5 µg of DNA, and 4 pmol of primers in a final volume of 12 µl of $dH_2O$. Oligo nucleotide primers used in the sequencing reaction were ordered from PE-Applied Biosystems UK. Results are sent by Send Express or e-mail and contain the sequence and a densitometric scan, which was viewed using the program Editview.

**2.3 Polymerase Chain Reaction**

PCR is the method used for the amplification of specific DNA sequences, using primers of known sequences. The PCR reactions were made up in a 0.5 ml microcentrifuge tube containing: 0.1 µl of cDNA (10 ng), 50 pmoles of forward

primer, 50 pmoles of reverse primer , 0.5 µl of Taq polymerase, 4 µl of dNTPs (50 µm), 1.5 µl $MgCl_2$ (50mM), 5 µl of 10x buffer (500mM KCl, 100mM Tris-HCl pH 9, and 1.0% Triton X-100), and 34.6 µl of $dH_20$. A layer of mineral oil was placed over the reaction mixture to prevent evaporation. The mixture was placed into a thermal cycler (MJ Research, Inc.) and run using a program called CK1 edited (step 1: denaturing at 94°C for 4 minutes, step 2: denaturing at 94°C for 1 minute, step 3: primer annealing at 60°C for 1 minute, step 4: extension at 72°C for 2.5 minutes, step 5: 24 times to step 2, step 6: 72°C for 10 minutes, and step 7: 4°C for indefinite hold). After the PCR reaction, DNA was analysed on an agarose gel and ligated into a vectors for protein expression and/or sequencing. Oligo nucleotide primers used in the PCR reaction were ordered from PE-Applied Biosystems UK and contained restriction sites for easy insertion into vectors.

**2.4 DNA Cloning into Vectors**

*2.4.1 Restriction digests*: PCR transcripts and library subclones were placed into various plasmids by ligating the DNA into open vectors. The vectors were first opened using specific restriction enzymes who's sites were contained within the multiple cloning sites of the vector. The enzyme had to be chosen with several factors taken into consideration. The restriction site must only exist in the area of the vector where the insert is to be placed. Also, a similar site must exist in the DNA planned for ligation into the vector. This was usually engineered into the primers for PCR, or in the recovered plasmid in library screening. Finally, the DNA must be inserted into a vector at a restriction site that will keep the sequence in frame. DNA

(1-3 μg) and a specific restriction enzyme (2-10 units) were placed into a 1.5 ml Eppendorf tube with 1 μl of the corresponding 10x buffer and water up to 10 μl. If more than one restriction enzyme was needed then the buffer was changed to either One-Phor-All Buffer Plus™ (Pharmacia) or Multi-Core™ (Promega). These buffers enabled different restriction enzymes to work in the same reaction tube, without having to modify the conditions. The tube was placed at 37°C for 1 hour and then run on a DNA gel.

*2.4.2 Ligation of inserts*: Ligation of inserts into vectors was accomplished by using the Ready-To-Go™ T4 DNA Ligase kit (Pharmacia). A ratio of insert DNA to linearized vector of about 5:1 was added to the reaction tube provided and made up to a final volume of 20 μl with $dH_2O$. The tube was incubated at 16°C for about 1 hour and the ligated plasmid was used directly from the reaction to transform competent cells.

*2.4.3 pBluescript®*: This vector (Stratagene) is a 2.96kb plasmid which was used to recover λZAP library screens and to place them into bacteria. The vector contains a lacZ gene, an IPTG-inducible lac promoter, ampicillin resistance and a polylinker region containing 21 unique restriction sites. The lac promoter allows "α-complementation" of cells containing a deletion in their lacZ gen (LacZ△M15) to produce a β-galactosidase protein. Production of this protein provides a blue/white colour selection. Production of active β-galactosidase protein cleaves the substrate X-gal, which produces a blue bacterial colony. If an insert has been placed between the two parts of the lacZ gene then this protein is not made, thus producing white colonies. DNA can then be amplified and used for many techniques, such as

restriction mapping, in vitro synthesis of RNA transcripts using the T3 and T7 bacteriophage promoters, DNA sequencing and fusion protein expression.

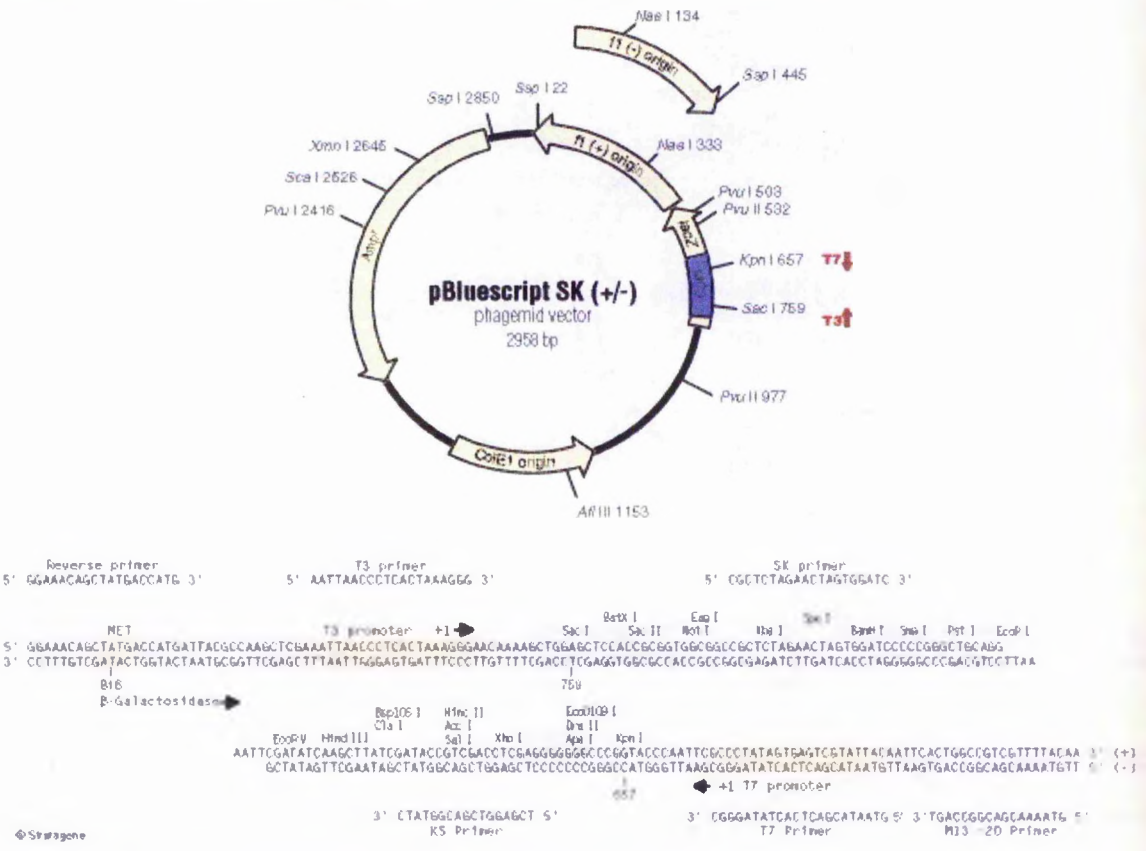



**Figure 8: pBluescript:** Ampicillin resistant plasmid, used for cloning and production of RNA. Contains many useful restriction sites and T3 and T7 bacteriophage promoters.

*2.4.4 pGEM-T®*: This vector (Promega) was a simple system for the cloning of PCR products. The vector has 3' terminal thymidine at both ends of the insertion site. They improve ligation by taking advantage of the addition of a single deoxyadenosine to the 3'-end of PCR products by certain polymerases. It also contains a β-galactosidase region for colour selection, T7 and SP6 RNA polymerase promoters, a f1 origin of replication, to produce single-stranded DNA, resistance to

100 μg/ml of ampicillin and 16 unique restriction sites. After insertion the plasmid was inserted into competent cells and amplified.

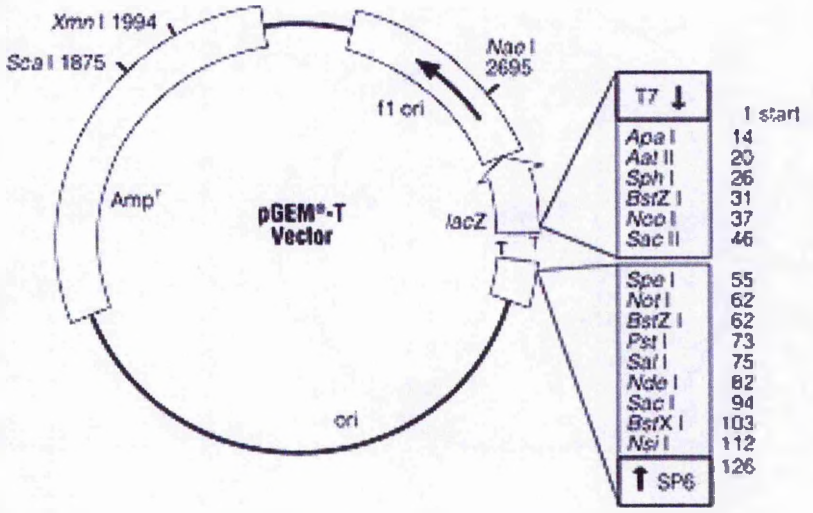



**Figure 9: pGEM-T:** Ampicillin resistant plasmid, used for amplification of PCR products. The vector contains many useful restriction sites and has 3' terminal thymidine at both ends to facilitate ligation.

*2.4.5 pGEX-4T®*: This vector (Pharmacia) is quite an efficient system for the expression and purification of fusion proteins. This plasmid is designed for inducible, high level intracellular expression of DNA with *Schistosoma japonicum* glutathione S-transferase (GST). The vector contains a tac promoter inducible expression for protein, an internal lac Iq gene for use in E. coli, resistance to 100 μg/ml of ampicillin, six unique enzyme cleavage sites, and a thrombin recognition site for cleaving protein from GST fusion. There are three different forms: 4T1, 4T2, and 4T3 which provides all three translational reading frames. Fusion proteins are induced with 0.1-0.5mM isopropyl β-D-thiogalactoside (IPTG) to produce the 26kDa GST and accompanying fusion protein.

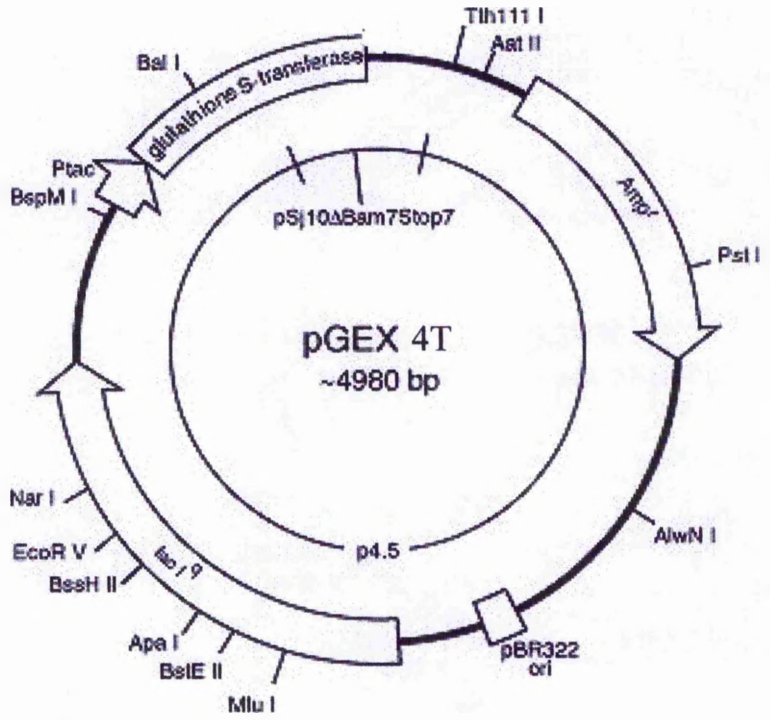



**Figure 10: pGEX:** Ampicillin resistant plasmid used for expression and purification of fusion proteins. The vector contains many useful restriction sites and contains a tac promoter inducible expression for protein.

*2.4.6 Transformation*: XL1-Blue and BL21(DE3)PlysS strains of *E. coli* were made competent for transformation by using the calcium chloride method (Sambrook *et al.*, 1989 p1.82). Overnight cultures were pelleted and washed in cold 0.1M $CaCl_2$. These cells were aliquoted into 200 µl amounts, into separate Eppendorf tubes and frozen until needed. Vector DNA (50 ng or less) was added to the 200 µl of competent cells and left on ice for 30 minutes. Infected cells were then heat shocked at 42°C for 1.5 minutes and then place one ice. 1 ml of LB was added and then it was placed into a water bath of 37°C for 1 hour. 200 µl samples were then transferred to

LB agar plates plus ampicillin. Transformed cells were evenly distributed over the plate using a glass spreader and placed overnight into a 37°C incubator.

**2.5 Fusion Protein Production**

GST fusion proteins were produced by pGEX-4T1, 4T2, or 4T3 vectors that had been transformed into XL-1 or BL21(DE3)PlysS cells. The transformed cells were stored on agar plates containing 50 μg/ml of ampicillin. Colonies were picked from these plates using a sterile toothpick and placed into a tube with 5 ml of LB and 50 μg/ml of ampicillin. Cultures of selected clones were grown up at 37°C overnight on a rotating wheel. The next day 2 ml of these overnight cultures were used to inoculate a flask containing from 200-500 ml of LB with 50 μg/ml of ampicillin added. This culture was grown at 37°C for 4 hours while air was pumped into the flask using a Whisper 600 air pump (Tetra). Expression was then induced by adding IPTG (usually 0.1-0.5 mM) and placing the culture at room temperature for an additional 4 hours. The induced cells were spun down and raised in 4 ml of TBS before freezing overnight. The next day the cells were sonicated, on ice, using a Sonifier® cell disrupter (Heat-Systems-Ultrasonics, Inc.). After sonication, 1 mM of the protease inhibitors: Phosphoramidon, Bestatin, Leucine Aminopeptidase, PMSF and E64(Sigma) and 0.5% lysozyme (Sigma) were added, plus 1% Triton X-100 (BDH Chemicals LTD). The sonicate was spun at 10,000 rpm for 10 minutes to separate the soluble protein from the cell pellet. This supernate containing the fusion protein was then bound to GST beads, following the protocol set out by the manufacture (Pharmacia). The purified fusion protein was eluted from the beads

using 10 mM reduced glutathione, after which it could be stored frozen or cleaved with Thrombin.

**2.6 SDS-Polyacrylamide Gel Electrophoresis**

Analysis of proteins were performed by running them on a 12% polyacrylamide gel, using the Hoefer "Mighty Small II System" for electrophoresis. Gels measured 10 x 8 cm and consisted of 4 ml of a separating gel and 1 ml of a stacking gel.

*2.6.1 The separating gel* consisted of 1.6 ml of 30% acrylamide, 0.5 ml of 1M Tris.$SO_4$ (pH 8.3), 1.3 ml of $dH_2O$, 0.57 ml of glycerol, 0.06 ml of 10% SDS, 8 µl of ammonia persulfate, and 6 µl of TEMED.

*2.6.2 The stacking gel* consisted of 0.2 ml of 30% acrylamide, 0.125 ml of 0.5M Tris.$SO_4$ (pH 6.9), 0.67 ml of $dH_2O$, 15 µl of 10% SDS, 3 µl of ammonium persulfate, and 3 µl of TEMED.

Both stacking and separating gel portions were de-gassed using a vacuum oven for 10 minutes. The separating gel was loaded first between a glass and aluminium plate using a 5 ml pipette, clamped in a gel caster, and allowed to polymerise for 15 minutes with a layer of $dH_2O$ over the top. The $dH_2O$ allows for a more efficient polymerisation and to ensure that the top of the separating gel was flat. The stacking gel was poured after the polymerisation of the separating gel was complete and a comb was inserted into the stacking gel so wells would form as it polymerised. The gel was clamped into electrophoresis chamber and a reservoir

buffer containing 3 g of Tris base, 14.3 g of glycine and 1.5 g SDS per litre was added to the top and bottom of the gel.

Higher and lower percentage gels were made by increasing or decreasing the amount of 30% acrylamide. Gels were made to 9% acrylamide for better resolution of proteins that were above 100kDa or 20% acrylamide for better resolution of proteins that were below 40 kDa. Protein molecular weight markers were obtained from Biorad or Sigma and consisted of unstained or prestained markers of broad range, high or low molecular weights.

Protein sample buffer was added to the sample protein and boiled for 2 minutes. The sample buffer consisted of SDS at 2% (w/v), 2-mecaptoethanol at 5% (v/v), glycerol at 10% (v/v) and Tris/$SO_4$, pH 6.9 at 10mM. The samples were loaded into the wells and run at 150 V for about 1.5 hours. After proteins had separated, the gel was stained in about 20 ml of stain, in a plastic box with gentle agitation, for about 30 minutes. The stain (500 ml) contained 1 gram of Coomassie brilliant blue, 50 ml of glacial acetic acid, 250 ml of 96% ethanol and 200 ml of $dH_2O$. After staining, the gel was destained to resolve the protein bands in two volumes of 100 ml of destaining solution for about 30 minutes each. Destaining solution contains 250 ml of 96% ethanol, 100 ml of glacial acetic acid and 650 ml of $dH_2O$. Destained gels were then dried down onto blotting paper, using a vacuum drier (Biorad), or placed into a sealed plastic bag.

## 2.7 Western Blots and Immunoblotting

Instead of staining gels to resolve individual proteins, gels could be transferred to nitrocellulose paper and used for Western blotting, using antibodies for specific proteins (ELISA or ECL). The gel was removed from the Hoefer "Mighty Small II System", after separation was completed, and was placed in a container with 100 ml of the same buffer as used in the reservoir (now called transfer buffer) for about 15 minutes. This is to allow the gel to expand and to wash away any particles that might interfere with the transfer. Nitrocellulose paper was then cut exactly to the same dimensions of the separating portion of the gel (6 cm, 8.5 cm) and also four pieces of 3M Whatman filter paper were cut to the same dimensions, and placed into a separated container with 100 ml of transfer buffer. After sufficient soaking in transfer buffer, the 3M paper was placed in a Hoefer TE 70 Series Semi-Dry Transfer Unit. Two sheets of 3M paper soaked with transfer buffer were stacked on the anode, and then the nitrocellulose paper was placed directly on top. The polyacrylamide gel was placed so the separating portion of the gel was in direct contact with the nitrocellulose paper. The other two pieces of buffer soaked 3M paper were placed over the top forming a sandwich. A plastic rod was rolled over the top of this sandwich to remove any air bubbles that might be between the gel and the nitrocellulose paper. The cathode portion of the transfer unit was placed on top and a current of 35mA was passed between the two plates for 2 hours. After the transfer, the nitrocellulose paper was removed and placed into a blocking solution of 10% skimmed milk (w/v) and Tris-buffered saline (TBS) made of 10mM Tris.HCl at pH 7.4 and 150mM NaCl. The transfer was allowed to block for 1 hour or overnight at 4°C.

*2.7.1 Immunoblotting using ELISA:* The enzyme-linked immunosorbent assay (ELISA) is a technique used for identifying specific protein bands, which have been transferred to nitrocellulose, by binding immunoglobulins covalently linked to an enzyme. Protein gels transferred to nitrocellulose were blocked in 10% milk in TBS overnight and then probed with a primary antibody specific for the protein. The serum containing the antibody was diluted in TBST, usually 1/1,000, and washed over the nitrocellulose transfer for 1 hour at room temperature. The transfer was washed at least six times in 20 ml of TBST to remove the unbound and non-specifically bound antibodies. The secondary antibody was then added to the transfer, usually at a 1/3,000 dilution. The secondary antibody was a goat anti-rabbit IgG which would recognise all rabbit antibodies bound to protein, and would subsequently bind to them. These secondary antibodies had a horseradish peroxidase enzyme conjugated to them. After binding with gentle agitation for 1 hour the transfer was washed several time in TBST and then finally in TBS. The proteins were detected by adding 100 µl of DAB (diaminobenzidine) and 3 ml of 30% hydrogen peroxide to 12 ml of TBS. This solution was washed over the transfer for about 1-2 minutes, until a colour reaction occurred representing a positive single for the protein band. The reaction was then stopped by washing the transfer in distilled water, in order to prevent the transfer from becoming too dark.

*2.7.2 Immunoblotting using ECL*: Enhanced chemiluminescence (ECL) is an extremely sensitive technique used for identifying specific proteins, using antibodies linked with the same HRP enzyme. The protocol is very much the same as ELISA, except after binding of both antibodies (primary IgG at 1/500 and secondary at 1/10,000), the transfer is treated with a fluid containing luminol which is oxidised by

HRP and hydrogen peroxide to produce light. The transfer is placed on X-ray film and left to expose for about 1 minute depending on the strength of the signal. The film was then developed by normal photographic procedures. The transfer can be re-probed with new antibodies after removing the antibodies with a stripping buffer (100mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5mM Tris-HCl pH6.7) while incubating it at 50°C for 30 minutes. It must be washed and re-blocked in 10% milk in TBS before it is ready to be re-probed.

**2.8 Northern Blots**

*2.8.1 Riboprobe synthesis and purification:* Various riboprobes were prepared by synthesising run-off transcripts, from appropriately linearized cDNAs, cloned into pBlueScript in the presence of [$\alpha$-$^{32}$P]CTP. The label was obtained from Amersham International at 400 Ci/mmol. Approximately 1-2 μg of DNA was digested in a total volume of 7 μl, using the appropriate restriction enzyme, at 37°C for 1-2 hours. Next, the following were combined: 7 μl of linearised DNA, 4 μl of 5 x salts, 2 μl of 0.1 M DTT, 1 μl of RNase inhibitor, 2 μl of 10 x NTP mix, 2 μl of [$\alpha$-$^{32}$P]CTP, 1 μl of T7 RNA polymerase, 1 μl of DEPC $H_20$ for a total reaction of 20 μl. The 10 x NTP mix contained: 10mM of ATP, 10mM of GTP, 10mM of UTP, and 0.5 mM of CTP. In a typical reaction, there was a 10:1 ratio of 1 nmol "cold" CTP to 0.1 nmol radiolabeled CTP. The reaction was incubated at 37°C for 1 hour, after which 1 unit of RNase-free DNase I was added and incubated for a further 10 minutes at 37°C. 30 μl DEPC $H_20$ was added before proceeding to a spin column. Radiolabeled RNA was recovered via the spin column containing Sephadex G-50 resin. This resin was

resuspended in TE buffer (20mM of Tris.HCl, pH 8.0 and 1mM of EDTA) and autoclaved. The column was prepared in a disposable syringe as described (Sambrook *et al.,* 1989): by plugging the syringe with some flock previously boiled in TE buffer, then filling it with the Sephadex G-50 suspension. The resin was compacted to dryness by spinning the column at 3,000 g for 4 minutes. 50 µl of TE buffer was added and spun through similarly in order to verify that the volume recovered was equal to the volume loaded. The riboprobe labelling mix was added and spun, un-incorporated nucleotides remaining in the column while the labeled probe was collected in a 1.5 ml screw-cap Eppendorf tube. The syringes and the collecting Eppendorf tubes were placed in an appropriate carrier centrifuge tube. The riboprobe was aliquoted into small volumes (usually 10 µl) and stored at -70°C.

*2.8.2 Extraction of RNA from oocytes:* Oocytes were collected in groups of 5-15 oocytes for vitellogenic stages and 50 for smaller previtellogenic oocytes and were placed in separate 1.5 ml microcentrifuge tubes. 500 µl of TNES buffer (100mM Tris.HCl, 300mM NaCl, 10mM EDTA, 2% SDS) and 200 µg/ml proteinase K were added to each tube, mixed thoroughly by vortexing, and incubated at 50°C for 60 minutes with further occasional vortexing. Samples were extracted twice with phenol-chloroform and once in chloroform. The RNA was precipitated by adding 2.5 volumes of ethanol and spinning at 10,000 rpm for 30 minutes at 0°C. The pellet was washed in 70% ethanol, resuspended in 20 µl of DEPC $dH_20$ and stored at -70°C. To extract RNA from embryos, a similar procedure was followed, however, the embryos were first placed for a short time in a solution of 2 % cysteine hydrochloride (pH 8.1)

to remove the jelly coating and rinsed four times in one third strength Modified Barth's Solution with gentle swirling.

*2.8.3 Northern blotting:* To every 10 µl of RNA solution (equivalent to the extract of 2 oocytes or embryos), 4 µl of 5 x MOPS (0.2M 3-(N-Morpholino) propanesulphonic acid, 50mM sodium acetate and 5mM NaEDTA pH 7.0), 7 µl of formaldehyde and 20 µl of deionized formaldehyde were added, giving a total volume of 40 µl. Immediately prior to loading onto the agarose gel, 4 µl of RNA loading buffer was added. The RNA gel was prepared by melting 0.9 g of agarose into 35 ml of DEPC $H_20$. Once the agarose had melted, 12 ml of 5 x MOPS was added and the gel mix cooled to 50°C. Next 13 ml of pre-warmed formaldehyde was added, and the gel was then poured, covered in 1 x MOPS buffer, loaded with 20 µl of each RNA sample, and run at 20V overnight. The RNA was transferred to a nylon membrane by vacuum blotting. Firstly, the gel was washed in $dH_20$ and soaked in 50mM NaOH for 3-5 minutes to hydrolyse the RNA partially. The transfer buffer consisted of 1 litre of 10 x SSC (standard sodium citrate: 175.3 g of NaCl, 88.2 g of Nacitrate, per litre, final pH 7.0), and was run for three hours. After the transfer, the membrane was rinsed in 2 x SSC for 5 minutes, air-dried, and the RNA cross-linked to the nylon membrane by UV irradiation, using a Spectrolinker set at optimal crosslink (120,000mJ/$cm^2$). As an alternative, the RNA samples could be transferred onto a membrane using a "slot-blot" apparatus.

*2.8.4 Hybridisation of RNA probe*: The membrane was placed into 50 ml of pre-hybridisation solution, containing 1% Blotto (10 g of Skimmed powdered milk, 0.2% sodium azide and $dH_2O$ to final of 200 ml), 1 x SSC and 2% SDS prepared with

DEPC $dH_20$, and pre-hybridised at 65°C for 2 hours. Next, the membrane was covered in 25 ml of fresh pre-hybridisation solution, containing the appropriate antisense riboprobe. The riboprobe was hybridised with gentle mixing overnight at 65°C. The next day, the filter was washed twice for 20 minutes at 65°C in 0.5 x SSC/0.1% SDS, and then similarly twice in 0.2 x SSC/0.1% SDS. If a high background labelling was received then the filter was washed further in 0.2 x SSC/0.1% SDS. The membrane was finally rinsed in 100 ml of the last wash solution and set up for autoradiography.

**2.9 Oocytes**

*2.9.1 Collection of oocytes:* Oocytes were collected from mature *Xenopus laevis* females by dissecting a portion of the ovary, after anaesthetising in a solution of 0.1% tricaine (MS222, Sigma) in water. The ovary was then treated with collagenase type I (0.2%) in $Ca^{2+}$ free OR2 medium (82.5mM NaCl, 2.5mM KCl, 1mM $MgCl_2$, 1mM $NaHPO_4$, 5mM HEPES, 0.05% PVP and NaOH to pH 7.8) for 1.5-2 hours on a rotating wheel at room temperature. The oocytes were washed four times in $Ca^{2+}$ free OR2 and twice in modified Barths' solution, with 1/200 of antibiotic and antimycotic solution (Sigma) added. Barths' solution contains 88mM NaCl, 1mM KCl, 2.4mM $NaHCO_3$, 0.82mM $MgSO_4$, 0.33mM $Ca(NO_3)_2$, 0.41mM $CaCl_2$, 7.5mM HEPES, and NaOH to pH 7.6. The oocytes were then left in modified Barths' solution and allowed to recover overnight, before any other procedures were carried out. This treatment removed the oocytes from the surrounding ovarian tissue and allowed isolation of individual development stages according to Dumont (1972).

Oocytes were used within two days of this procedure or were stored at -70°C until needed.

*2.9.2 Isolation of nuclei:* Oocytes from stages III-VI were separated into individual dishes in Barths' solution. The oocytes were taken up in a glass Pasteur pipette and placed on 3M blotting paper at the bottom of a 30 mm petri dish. The buffer was soaked up by the 3M paper and 2.5 ml of paraffin oil was poured over the top of the oocytes. The dish was placed under a dissection microscope and a small hole was made at the animal pole with a fine tungsten needle. The nucleus of the oocyte slowly came to the top and it was taken off using a 20 µl pipette and placed into a 1.5 ml microcentrifuge tube. Nuclei were collected into 20 µl of kinase buffer (20mM Tris-HCl pH 7.4, 5mM MgCl, 1mM DTT, and 100mM KCl) in 50% glycerol if their activity was to be measured, or 20 µl of homogenisation buffer (0.1M KCl, 20mM Tris-HCl pH 7.5, 2mM MgCl, 0.2% NP40 and 2mM DTT) if they were to be run on a protein gel. On average about 10-50 nuclei were collected at one time. The microcentrifuge tube was spun at 500 rpm on a microcentrifuge for 1 minute, so that nuclei would separate from the oil phase into the buffer phase. The paraffin oil was then removed using a 200 µl pipette and the nuclear extract was stored at -20°C. This procedure is similar to the method used by Pain *et al.* (1992).

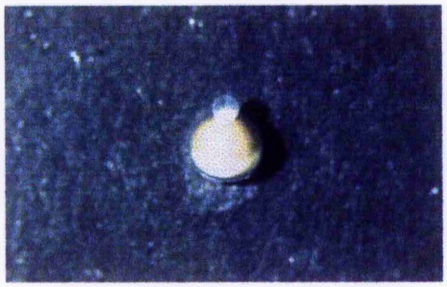


**Figure 11: Isolation of Nucleus:** A stage VI *Xenopus* oocyte, under oil, with a protruding nuclei at the animal pole.

*2.9.3 Isolation of cytoplasms:* The cytoplasms that were left, after extraction of the nuclei, were collected and placed into 60 µl of homogenisation buffer in a 1.5 ml microcentrifuge tube and spun at 500rpm on a microcentrifuge for 1 minute. This was done to ensure that the cytoplasms would separate from the oil phase and passed into the buffer phase, after which the oil was then removed. The cytoplasms were then homogenised by pipetting the mixture up and down using a 20 µl pipette. An equal volume of Freon (1,1,2-trichlorotrifluoro-ethan, Sigma) was added to the homogenate, and thoroughly mixed by vortexing. The homogenate was then spun at 10,000 rpm for 10 minutes at 4°C in an Sorvall swing-out rotor. The supernatant containing the solubilized protein was removed using a 200 µl pipette and the Freon containing the lipid and yolk was discarded. The cytoplasmic extract was placed into a clean microcentrifuge tube and stored at -20°C.

## 2.10 Glycerol Gradients

Gradients were made by using a linear gradient maker containing 25% and 10% glycerol in a gradient buffer (100mM KCl, 20mM Tris-HCl pH 7.5, 2mM MgCl, 1mM DTT and 0.1% NP-40). Protein samples were prepared in homogenisation

buffer, in a microcentrifuge tube and spun at 10,000 rpm for 10 minutes, after which the supernatant was removed. Samples could also be treated with a non-ionic detergent, such as 0.1% NP-40, or with Freon. This supernatant was added to the top of the gradient using a pipette and placed in SW65 rotor of the Beckman L-7 ultracentrifuge. The samples were spun at 30-36,000 rpm for 16-18 hours at 0°C. Samples of 300 µl were collected by carefully pipetting from the top of the gradient. Proteins were precipitated from gradient fractions by adding 900 µl of acetone to each 300 µl sample. It was left on ice for 30 minutes and then spun at 10,000 rpm for 25 minutes at 4°C. The supernatant was removed and the pellet was air dried for 10 minutes. The pellet was raised in 10 µl of 8M urea and 10 µl of SDS-PAGE buffer was added prior to loading on a protein gel. Proteins were analysed using immunodetection.

**2.11 Isolation of Protein Kinase Activity and Phosphorylation of Proteins**

*2.11.1 Nuclear protein kinase*: Nuclei were isolated using the procedure described in the "Oocyte" section under "Isolation of Nuclei". Nuclei were isolated into kinase buffer and 50% glycerol. The nuclei were spun on a microcentrifuge for 1 minute and any paraffin oil was removed from the top. The nuclei were broken up by pipetting with a 20 µl micropipette fitted with a white micropipette tip. This homogenate was stored at -20°C until needed.

*2.11.2 Kinase extract*: Kinase activity was purified from isolated nuclei by binding the nuclear extract to heparin-Sepharose (Pharmacia) and then washing off the bound protein kinase. 50 nuclei were isolated in 50 µl of protein kinase buffer

plus 50% glycerol, using the protocol described in the "Oocyte" section under "Isolation of Nuclei". This was diluted with 200 µl of TBS and 40 µl of heparin-Sepharose was added to the mixture. The enzyme was allowed to bind over several hours at 4°C on a rotating wheel. The unbound nuclear extract was removed and the resin was washed four times with ~1 ml of TBS on ice. The resin was then washed with 1 ml of 0.2M KCl to remove any partially bound protein. The kinase was eluted in a 200 µl of 1M KCl. Glycerol was added to 50% and it was stored at -20°C.

*2.11.3 Poly (A+) RNA-associated protein kinase*: The poly (A+) mRNP was isolated using the same methods as described in the "poly (A+)" section. The associated protein kinase would be isolated by affinity chromatography using heparin-Sepharose, as described above, and was stored at -20°C.

*2.11.4 Fusion protein phosphorylation:* GST-fusion proteins were bound to 100 µl of Glutathione Sepharose 4B beads and washed four times with 1x kinase buffer. About 8 µg of these bound proteins in 78µl was added to 10 µl of 10x kinase buffer, 10 µl of nuclear extract (Nuclei, heparin bound, or A+) and 1 µl of [$^{32}$P] γ-ATP (10µCi)(0.033mM). This mixture was incubated for 1 hour at room temperature. Beads were washed four times in TBS and then phosphorylated protein was removed using 10mM reduced glutathione plus 2% n-Octyl β-D-Glucopyranoside (Sigma).

**2.12 Sequencing of Phosphorylated Protein**

GST fusion proteins were labelled using [$^{32}$P]γ-ATP and then digested in trypsin (20 µg/ml) in .01 M ammonium bicarbonate at 37°C for 4 hours. The sample

was raised in 50µl of 0.1% trifluroacetic acid and 20 µl of this was applied to a C-18 microbore column. The elution gradient used was a 5 minute isocat step of 5% acetonitrile, followed by a gradient to 50% acetonitrile over 30 minutes and a 19 minute rise to 95%. The fractions of 80 µl each were collected every minute and 2 µl aliquots were spotted on to filter paper and assayed overnight for radioactivity using a phosphoimager. The peak radioactive fractions were lyophilised and resuspended in 10 µl of 30% acetonitrile. These samples were spotted on to a half disk of Sequelon-AA (Millipore) membrane (PVDF derivitised with aryl groups) and dried at 55°C on a heating block. The dried peptide was then attached to the membrane by addition of 5 µl of 10 µg/ml solution of carbodiimide (EDC). After leaving at room temperature for 20 minutes, the membrane was washed 3 x in 0.5 ml of 50% methanol and then placed in the sequencer. The extracted ATZ derivative from each sequence cycle was collected, dried and resuspended in 10 µl of 90 % methanol. Aliquots of 2 µl were spotted on to filter paper and assayed overnight for radioactivity using the phosphoimager (Llinas *et al.*, in preparation). This experimental procedure was carried out by Dr. Graham Kemp of St. Andrews University.

**2.13 Microinjection of Protein**

*2.13.1 Preparation of injected protein*: 40 µg of fusion protein, isolated from GST beads, was diluted with 0.5 ml of $dH_2O$ and placed into a Filtron centrifugal concentrator. The mixture was spun on a Sorvall RC-5B centrifuge, at 8,000 rpm for 30 minutes at 4°C, until a volume of 10 µl was recovered and adjusted to 20mM

sodium phosphate (pH 7.2). A 1/20 volume of neutral red (saturated) stain was added to provide colour for the injected solution.

*2.13.2 Injection of protein into oocytes*: Oocytes were collected and deposited on a 1 mm mesh in a 300 mm Petri dish, containing Barths' solution. 20-50 nl of protein was injected into each oocyte's vegetal pole, using a 25 μm micropipette. The pipette was housed in a Algal syringe attached to a micrometer screw, which when turned would force air from the end of a Teflon line filled with paraffin oil. Thus, the protein was injected by increasing the pressure behind the injectant. The protein was loaded into the micropipette by decreasing the pressure, thus bring the protein into the micropipette. After the injection, the oocytes were left sitting in clean Barth's solution, and samples at different time points were taken to measure the import of different proteins into the nucleus. Samples were analysed by running them on protein gels and immunostaining them, or they were dried down and set up for autoradiography.

**2.14 Antibody Production**

Fusion proteins (CK2α and CK2β) were grown and purified using GST beads. A solution containing 0.4 mg of purified protein, in 0.5 ml of TBS, was emulsified by combining it with 0.5 ml of the oil "Drakeol 6VR", (Pennsylvania Refining Company) which contained 1/10 volume of the emulsifier Arlacel A (Atlas). 1ml of 2% Tween 80 (Sigma) in 0.14M NaCl was added and mixed using a 26 gauge needle on a 2 ml syringe. This emulsion was injected subcutaneously into Dutch rabbits and a test bleed was taken from the marginal ear vein. At six weeks a second emulsion was injected, identically as the first as a booster and bleeds of 10 ml were

collected every two weeks. A second booster was sometimes necessary to increase the titre level of antibodies. This was accomplished by injecting 0.2 mg of fusion protein, in 0.3 ml of TBS, into the marginal ear vein. Blood samples were taken and allowed to coagulate at room temperature for 1-2 hours. The serum was then removed and spun at 5,000g for 10 minutes to remove white blood cells, then aliquoted and frozen at -70°C. This serum could be used directly for immunoassays, or purification of IgG could be performed. 1 ml of serum was incubated with 250 µl of protein A-Sepharose beads for 1 hour at 4°C with gentle agitation. The beads were washed four times with 0.1M borate buffer (pH 9), to remove serum protein not bound to the beads. IgG was eluted from the beads using 0.1M citrate (pH 3), and then neutralised by adding one-tenth volume of 1M Tris-HCl (pH 9).

**2.15 Electronic Autoradiography**

The InstantImager® (Packard) is a fully automated quantifier of radioactivity. It is computer controlled sensor and recorder of different types of flat radioactive sources. Samples could be labelled with Phosphorus-32, Phosphorus-33, Carbon-14, Iodine-125 and Sulphur-35. Protein gels that contained protein, labelled with one of these sources, were dried down on a piece of 3M paper and placed into the imager's sample drawer on the sample plate. A the sample cover would be placed over the gel and the drawer closed. The Imager window would then be opened and the user would start the "Acquisition" cycle. Depending on the amount of radioactivity, an image would be obtained in a very short time compared to imaging on to film. Exact procedures were followed from the Instantimager user manual. Once the image was

recorded by the computer, the user could print out a picture and/or measure the exact amount of radioactivity in each part of the gel. The system comes with software which allows the user to display the image, annotate the image with text and shapes, analyse the image by creating a template and produce a report of all quantitative results specified by the user.

### 2.16 Cell Culturing

XTC cells are a late embryonic cell line first cultured from *Xenopus laevis* tadpoles in 1969 (Pudney *et al.*, 1973). They are epithelial in nature and have a rapid growth rate, which makes them ideal for cell culturing. Cells were cultured according to protocols from "Methods of Cell Biology" (Smith and Tata, 1991). Cells were grown in a 500 ml culture flask, containing 50 ml of modified Leibovitz L-15 medium, in a 25°C incubator. The cell culture medium contained 61% L-15, 27% sterile $H_2O$, 10% fetal calf serum, 1% Pen-Strep (10,000 u/ml), and 1% L-glutamine (200mM). Once cells had reached confluence they were split into separate flasks or collected for experimental use. Cell cultures would be split by treating them with trypsin (8 µl trypsin, 20 ml 0.2M EDTA (pH 8)), and 2 ml calcium-free medium (88mM NaCl, 1mM KCl, 2.4mM NaHCO3 and 7.5mM Tris (pH 7.6)). These procedures were carried out in a flow cabinet under sterile conditions.

### 2.17 Tissue Section Immunostaining

*2.17.1 XTC cells:* XTC cells were prepared for staining by growing them on glass cover slips in 90 mm plastic petri dishes. After the cells had reached confluence the cover slips were removed from the petri dish and placed into a fresh one. They

were washed with clean Lebowitz solution to remove dead cells. The coverslips were placed for 10 minutes into 100 ml Columbia jars filled with methanol at -20°C to fix the XTC cells. They were then transferred to another flask containing acetone at -20°C for another 10 minutes. The cover slips were air dried for 2 minutes and then washed twice in PBS (137mM NaCl, 3mM KCl, 15mM $KH_2PO_4$, 7mM $Na_2HPO_4$). The coverslips were placed in PBS, plus 5% fetal calf serum, and left for one hour to block. After blocking was complete the coverslips were placed in PBS, plus 1% fetal calf serum, with CK2α antibody at 1/200 dilution and incubated at room temperature for 1 hour. They were washed after the primary antibody binding in PBS five times for 10 minutes each. The secondary antibody was then added, a Fluorescein Isothiocyanate conjugated anti-rabbit IgG (FITC). This was added at 1/500 dilution in PBS, plus 1% fetal calf serum, and incubated in the dark for 1 hour. Again, after the binding was completed, the coverslips were washed five times with PBS, plus 1% fetal calf serum, for 10 minutes each. The coverslips were removed from the final wash and placed face down onto a microscope slide with a drop of mounting solution (50mM Tris-HCl pH 8, 1.5mg/ml of n-propylgallate, 50% glycerol, 20μg/ml of DAPI(4,6-Diamidino-2-phenylindole, Sigma)). The stained cells were examined with a fluorescence microscope.

*2.17.2 Ovary sections:* Previtellogenic ovary was dissected from immature *Xenopus* and then fixed for 1 hour with a solution containing 3% paraformaldehyde and 0.25% glutaraldehyde in 0.1M PBS. Afterwards they were washed in PBS, plus 0.1% sucrose, for 10 minutes. They were place in 70% ethanol and allowed to sit overnight at 4°C. The tissue was then rehydrated, embedded in wax and 7 micrometre

sections were taken and attached to microscope slides. Before immunostaining could begin the sections had to be de-waxed. This was done by washing them in the solvent Histoclear two times, then absolute alcohol, 96% alcohol, 70% alcohol, and then finally in PBS. The immunostaining was carried using the same protocol as with the XTC cells.

**2.18 Isolation of mRNP Particles and Y-Box Proteins**

*2.18.1 mRNP:* Previtellogenic, or early vitellogenic ovary (~0.5g), collected from immature *Xenopus laevis*, was sonicated in 2 ml of column binding buffer (0.25M NaCl, 2mM $MgCl_2$, 1mM DTT, 0.2% NP-40, 20mM Tris-HCl (pH 7.5)). The homogenate was then centrifuged at 10,000 rpm for 15 minutes at 2°C. The supernatant was removed, NaCl was added to a final concentration of 0.25M, and passed through a 1.5 ml Oligo(dT)-cellulose column (Pharmacia) at 4°C. The column had been pre-washed with the column binding buffer. The unbound material was passed though the column three times to ensure complete binding of poly (A+) mRNP. This was monitored by measuring with $A_{254}$, which indicates RNA content. The unbound material (poly(A)-) was collected, and the column was washed thoroughly to remove unbound material with binding buffer, until the $A_{254}$ returned to background levels. The poly (A+) mRNP was eluted from the column in 2.5 ml of warm $dH_2O$ and stored at -70°C. This procedure is similar to the method used by Cummings *et al.* (1989).

*2.18.2 Y-box proteins*: Heat treatment (Deschamps *et al.,* 1991) was used to purify Y-box proteins from an SN10, of previtellogenic ovary homogenised in HB

buffer minus NP40. The SN10 was heated to 80°C for 5 minutes, during which most proteins were denatured. The sample was then left to cool on ice for 10 minutes, during which most protein precipitated, but the Y-box proteins were able to refold, thus remaining soluble. The sample was spun at 10,000 rpm for 10 minutes and the supernatant was removed which contained the purified Y-box proteins. The heat treatment and cooling steps were repeated for a total of four times to ensure most proteins came out of solution, except for the Y-box proteins. The sample was then precipitated using 4 volumes of acetone. This was left on ice for 30 minutes and then spun at 10,000 rpm for 20 minutes. The supernatant was discarded and the pellet was raised in 8M urea.

**2.19 Antibody Affinity Binding**

Antibody affinity binding was done using a standard protocol (Glover and Hames, 1995). Antibodies were incubated with protein A beads (Prosep-A high capacity, Bioprocessing) and then cross-linked to the beads using dimethyl-pimelimidate dihydrochloride. After antibody beads were made, proteins from oocyte extract would be bound to the beads using a column chromatography protocol. Proteins were eluted from the antibody beads using elution buffers containing high salt concentrations (usually 3.5M $MgCl_2$) or low pH levels (usually pH 1.5-3.0). This technique was used to purify specific proteins or to study protein-protein binding of related proteins.

**2.20 Histone Deacetylase Activity Assay**

Histones (2 mg) purified from rat liver, or peptide representing the terminal eighteen amino acids of H4 (1mg), were dissolved in substrate buffer (0.5 ml of 50mM Na borate pH 9.0) and subsequently mixed with 6mCi of $^{3}$H-acetic anhydride. The mixture was incubated for 3 hrs. at 0°C, after which it was adjusted to 0.25M HCl and precipitated with twelve volumes of acetone, washed and vacuum dried. The activity of histone deacetylase was assayed using the methodology from Li *et al.* (1996). Nuclear extract (100µl) was mixed with 150µl of assay buffer (25 mM Na phosphate/citric acid pH 7.0), 20µl of $^{3}$H-acetylated peptide or histone mix (~1.2 x $10^{6}$dpm, dissolved in assay buffer) and $dH_2O$ up to 300µl. This was incubated for 1 hour at 37°C, after which the reaction was stopped by adding 0.12M of acetic acid and 0.72M of HCl and 2 ml of ethyl acetate. The samples were centrifuged at 9000 x g for 1 minute and then half the volume of acetate was removed and the dissolved $^{3}$H-acetate was measured by scintillation counting.

# Chapter 3: Results

# Protein Kinase CK2 from *Xenopus Oocytes*

## 3.0 Aim

The aim in this chapter is to study the expression and to define the activity of protein kinase CK2 in oocytes. Specifically, the amounts of protein expressed in different stages of oocyte maturation are examined, and levels in the nucleus and the cytoplasm are compared. The activity of the enzyme was measured using different substrates. Addition of selective inhibitors and various buffers was used to study the activity of this phosphorylating enzyme. As well as expression and activity, the particle size of the enzyme was calculated using glycerol gradients to determine if it is most active in its tetrameric form, and if smaller or larger units exist in oocyte nuclei.

## 3.1. Cloning and Expression of the *Xenopus* CK2 α-Subunit

*3.1.1 Design of PCR primers*: The first clone obtained was the product of a PCR reaction using synthesised primers to a region of the known *Xenopus* α-subunit sequence. The forward primer was 25 nucleotides long 5'[GACTGGGGCTTGGCCGAATTCTACC]'3 which represented the α-subunit from nucleotide 523 to 547 (Jedlicki *et al.,* 1992). The primer had a 60% GC content which gave it a melting temperature of 60°C. This sequence encodes a conserved region of the catalytic domain: 175-D W G L A E F Y H-183. The reverse primer was 27 nucleotide long 5'[CTGATGGTCATATCGCAGCAGCTTGTC]'3 and represented the α-subunit from nucleotide 904-930. The primer had a 52% GC content which gave it a melting temperature of 59°C. This sequence encodes a region close to the carboxyl end of the subunit: 302-D K L L R Y D H Q-310.

3.1.2 *PCR reaction*: The aforementioned primers were added together with the necessary PCR reactants (see Methods, section 2.3) and 10ng of single stranded cDNA (made from isolated *Xenopus* mRNA) which were then placed into the PCR apparatus for 25 cycles taking a total of 3 hours. After the PCR product was made and isolated from an agarose gel (see Methods, section 2.1.9) it was inserted into the pGEM-T vector (see Methods, section 2.4). This vector was used because it contains a 3' thymidine at each end of its insertion site which increases the efficiency of ligation of the PCR products. XL-1 cells were transformed with the recombinant vector (see Methods, section 2.4.6), clones were selected and the plasmid DNA was checked.

*3.1.3 Sequencing and cDNA screening*: The DNA was sequenced by Dr. Sommerville using standard manual DNA sequencing methodology. This was to ensure that no errors had occurred during the PCR and ligation process. This DNA was used as the template to make a radioactive probe in order to screen a cDNA library for a longer clone (see Methods, section 2.8.1). The cDNA library was screened (see Methods, section 2.1) and the positive clone, named 2A, was picked and purified. This resulted in a clone very similar to the one that was generated using the PCR reaction. It was somewhat different in length than that of the previous clone; the new one started from 180-glutamic acid (E) and ended at the 3' poly (A) tail (350-valine (V)). Since it was extracted from a lambda Zap cDNA library, the clone was in the pBluescript vector which made it very easy to amplify and purify the DNA.

**gac tgg ggc ttg gcc gaa ttc tac** cac cca gga cag gag tac aat gtc cgt gtt gct tcc cga tac ttc -591
D W G L A E F Y H P G Q E Y N V R V A S R Y F -197
aag ggg ccg gag ctg ctt gtg gat tac caa atg tac gac tac agc ctg gac atg tgg agc ctg ggg tgc atg ctg -666
K G P E L L V D Y Q M Y D Y S L D M W S L G C M L -222
gcc agt atg att ttc aga aaa gag cca ttt ttc cat ggc cat gat aac tac gac cag ctg gtg aga ata gcg aag gtg -744
A S M I F R K E P F F H G H D N Y D Q L V R I A K V -248
ctg ggc aca gaa gac ctc tac gat tat att gac aag tac aac ata gaa ctg gat cca cgc ttt aat gat att ctg ggc -822
L G T E D L Y D Y I D K Y N I E L D P R F N D I L G -274
agg cac tcg cgc aaa cgg tgg gaa aga ttt gta cac agt gag aac caa cat cta gtc agt cct gag gca ctg gat -897
R H S R K R W E R F V H S E N Q H L V S P E A L D -299
ttc ctg **gac aag ctg ctg cga tat gac cat cag** acc cga ctg act gcc cgt gaa gct atg gac cac cca tac ttc -972
F L D K L L R Y D H Q T R L T A R E A M D H P Y F -324
tat ccc atc gta aag gac cag tcc cgg atg gcg gct cta ata tgc cca gtg gca gca cac ccg tca gta gcg cca -1047
Y P I V K D Q S R M A A L I C P V A A H P S V A P -349
gta tga TGTCAGGTCAGTCCCGGCCAGTGTTCGTG -1082
V * -350

**Figure 12: CK2α clone 2A**: Nucleic and amino acid sequence of the CK2α clone 2A. The forward and reverse primers are in bold. * represents the stop codon in the open reading frame. Numbering from Jedlicki *et al.*, 1992, accession number p28020.

*3.1.4 Expression*: The 2A insert was removed from the pBluescript vector and ligated into a pGEX vector (see Methods, section 2.4). This is an expression vector which allows stimulation of the synthesis of a protein from the cloned cDNA, which is fused with glutathione-S transferase. The insert was removed from pBluescript by cutting it with the restriction enzymes EcoRI and XhoI, after which the DNA was isolated from an agarose gel. This DNA was then ligated into linearised pGEX-4T2 cut with the same restriction enzymes to enable proper ligation. XL-1 bacteria were transformed with the pGEX + DNA vector. Once the transformation was complete the protein was expressed with the addition of IPTG to the multiplying bacteria which carried the new vector (see Methods, section 2.5). The GST fusion protein was measured on SDS-PAGE at 46kDa: 26 kDa for GST and 20kDa for the fragment of the α-subunit. This fusion protein was purified and frozen for later use.

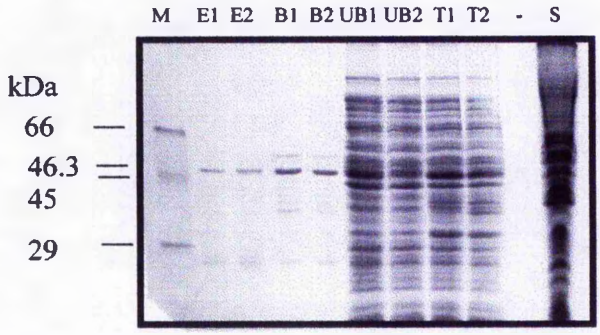



**Figure 13: Bacterial expression of CK2α clone 2A:** M represents the marker track containing markers of 66, 45 and 29 kDa. E1 and E2 are elutions of fusion proteins from glutathione-Sepharose beads, the fusion protein is 46.3 kDa. B1 and B2 are the beads with fusion protein still bound to them. UB1 and UB2 represent the unbound supernatant that did not bind to the beads. T1 and T2 are the total induced extracts before binding to the beads. S represents the sonicated whole pellet fraction.

*3.1.5 Antibody production*: Antibodies were produced against the clone 2A by injecting the purified fusion protein, expressed by the pGEX vector, into rabbits (see Methods, section 2.14).

### 3.2 Cloning and Expression of the *Xenopus* CK2 β-subunit

*3.2.1 Design of PCR primers*: The clone for the β subunit was the product of a PCR reaction similar to the one for the α-subunit. The forward primer was a 20 nucleotide long sequence which was 5'[GATGAAGACTATATCCAGGA]'3 and represented the β-subunit cDNA from nucleotide 76 to 96 (Jedlicki *et al.*, 1992). The primer had a 40% GC content which gave it a melting temperature of 46°C. This sequence encodes a region near the amino end of the protein: 26- D E D Y I Q D-32. The reverse primer was 21 nucleotides in length and had the sequence 5'[CCCC**CTCGAG**TTTTTTTTTTT]'3 corresponding to the linker ligated to the 3' end of the cDNAs of the lambda Zap library. This primer had a GC content of 38%

thus giving it a melting temperature of 52°C; it also has an engineered internal XhoI site for easy ligation.

*3.2.2 PCR reaction*: The PCR reaction was similar to that for the α-subunit. The product was purified by running on a agarose gel and then ligated into pGEM-T.

*3.2.3 Sequencing*: The clone was this time checked by the Sequencing Unit in the Irvine building of St. Andrews University. The sequence acquired contained most of the β-subunit coding region and the entire 3' UTR to the poly A tail.

**gat gaa gac tat atc cag ga**t aag ttt aac ttg aca gga ctc aat gag cag gtc cca cac tat aga cag gca ctg gac -153
D E D Y I Q D K F N L T G L N E Q V P H Y R Q A L D -51
atg ata ctg gat cta gaa cct gat gag gaa ttg gaa gat aat ccc aat caa agt gac ctc att gaa caa gct gca gag atg -234
M I L D L E P D E E L E D N P N Q S D L I E Q A A E M -78
ctg tat gga cta atc cat gcc cgt tac ata ttg act aac cgt ggc att gct caa atg ctg gag aaa tat caa cag gga gat ttt -318
L Y G L I H A R Y I L T N R G I A Q M L E K Y Q Q G D F -106
ggc tac tgt ccc aga gta tac tgt gag aac cag ccc atg cta cct att ggt ctc tca gat atc cct ggg gaa gct atg gta -399
G Y C P R V Y C E N Q P M L P I G L S D I P G E A M V-133
aaa ctg tat tgt ccc aag tgt atg gat gtt tac aca ccc aaa tcc tca cgg cat cat cac acc gat gga gca tat ttt ggc -480
K L Y C P K C M D V Y T P K S S R H H H T D G A Y F G-160
act gga ttt cct cac atg ctt ttt atg gtt cac cct gag tat agg ccc aag agg cct gcc aat caa ttt gtt cca agg tta tat -564
T G F P H M L F M V H P E Y R P K R P A N Q F V P R L Y -188
ggc ttc aaa atc cac cct atg gca tac cag ctt caa cta caa gca gcc agc aac ttt aaa agt ccc gtg aag acc atg cgt -645
G F K I H P M A Y Q L Q L Q A A S N F K S P V K T M R -215
tgaGTCTTCCTATTATATGGATTATACATGGGTCTGCACACCCCATAAACCCTATTTCCT
*
TCCATCGACAATGAAGATGTCGTTGCAGTGTATTATTTGCCCACAATTTTGATTTTA
GTCCTGTTAAAGTGTATTCCTGCAATTTCCAGTTGTGGTTAAATAAAATTCTAAAGA
ATCGTT (poly (A) tail).

**Figure 14: CK2β clone** β: Nucleic and amino acid sequence of the CK2β clone β. The forward primer is in bold. The * represents the stop codon in the open reading frame. Numbering from Jedlicki *et al.*, 1992, accession number p28021.

*3.2.4 Expression*: The insert was removed from pGEM-T by cutting with NcoI and XhoI and was ligated into pGEX-4T2 cut with Sma I and XhoI. The fusion protein was expressed similarly to the expression of the α-subunit clone. The β-subunit fragment fused with GST measured 48kDa: 26 kDa for GST and 22kDa for the β-subunit.

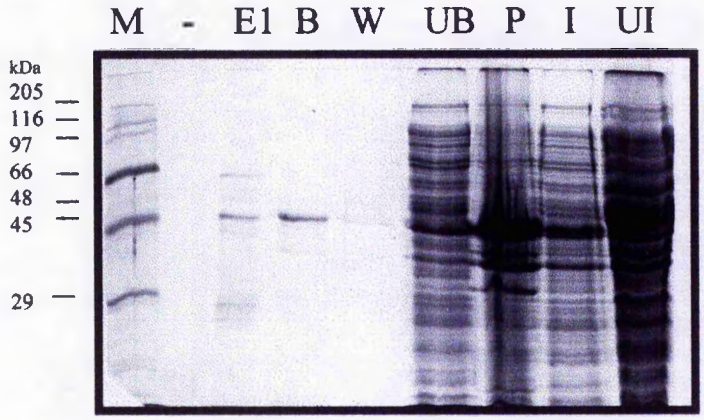



**Figure 15: Bacterial expression of CK2β clone β:** M represents marker track containing markers of 205, 116, 97,66, 45 and 29 kDa. E1 is the elutions of fusion proteins from glutathione-Sepharose beads; the fusion protein is 48 kDa. B is the beads with fusion protein still bound to them. W is the pool of the wash fractions to see how much of the fusion protein is removed before the elution. UB represent the unbound supernatant. P represents sonicated whole pellet fraction. I is the induced supernatant and UI is an uninduced supernatant for comparison.

*3.2.5 Antibody production*: Fusion protein was purified and injected into rabbits but no usable serum was collected. Although anti-GST activity was obtained, no reactivity was detected with native protein extracts. This may be due to the fact that there is such sequence homology between species, that the rabbit did not recognise this fusion protein as foreign, and so did not produce antibodies against it. Rabbits were boosted many times and also different rabbits were injected with shorter forms of the clone, however, no useful serum was obtained.

### 3.3 mRNA Expression

Several attempts were made to probe for the amount of mRNA expressed during the different developmental stages, using Northern-blotting (see Methods, section 2.8). These attempts were unsuccessful due to the probable instability of the message. Measurements of the amounts of mRNA were made by Wilhelm *et al.* in

1995, but they did not use Northern-blotting techniques; instead they used a competitive reverse PCR method to estimate the amounts of mRNA in each stage. This technique is less direct, suggesting that they had similar difficulty probing for this particular mRNA, especially when they had used the Northern-blotting technique to measure amounts of the same message in tissues.

### 3.4 Identification of the α-Subunit During Oogenesis

*3.4.1 Oocyte expression of native CK2α* : The serum made against the α-subunit clone 2A in rabbits was used to study protein kinase CK2 in *Xenopus* oocytes. This serum recognised both the α and α' subunits of 42 and 38 kDa from oocyte extracts.

Oocyte proteins were solubilized by bursting approximately 50 oocytes in homogenisation buffer which was then extracted with Freon to removed lipids. Extracts were then spun at 10,000 rpm to remove the soluble layer from the Freon, yolk, pigment and lipid phase (see Methods, section 2.9). A sample of this soluble protein was mixed with SDS-PAGE buffer and run on a protein gel (see Methods, section 2.6). The proteins were then transferred to nitrocellulose paper and blotted with the antibody (see Methods, section 2.7).

The level of CK2 α subunit increases in growing oocytes. The amounts of soluble CK2 can be seen to be increasing from its lowest levels in stage I, to a peak in stage VI of oogenesis (fig. 16A). The enzyme activity was reported to increases about 10 fold from stage I to stage VI (Wilhelm *et al.*, 1995) which parallels the increase of soluble protein detected here. Levels in nuclei compared to cytoplasms were also measured using the same antibodies, and a similar increase in signal can be seen through oogenic stages (fig. 16B). The amount of CK2α protein in each sample is

difficult to estimate since there are large amounts of total cellular proteins, preventing identification of specific bands on a Coomassie-stained gel. The ECL and DAB immunoblotting reactions were not quantitated. One significant fact is that there is a substantial amount of protein in the nucleus (fig. 16B). Although there are more nuclei added to this gel then cytoplasms, a ratio of 5 to 1, this is due to the much larger amounts of proteins recovered from the cytoplasms, which if added at equal ratios would overload the gel. Since the volume of the nucleus is much smaller than that of the cytoplasm (approximately $1/50^{th}$), it is clear that there is a higher concentration of CK2α in the nucleus compared to the cytoplasm. This supports the theory that CK2 is required more for nuclear functions which suggests that after translation in the cytoplasm most of it is shuttled into the nucleus.

In order to visualise CK2 in situ, ovary tissue sections were stained with anti-CK2α and a secondary fluorescent antibody (FITC-conjugated anti-rabbit IgG) was used to locate the primary antibody (see Methods, section 2.17) Ovary tissue staining of immature oocytes with anti-CK2α showed high levels of enzyme associated with the nuclear envelope as well as some staining of nucleoli (fig. 17). This would also lend support to the proposed nuclear compartmentalisation of CK2 and to the proposed role in ribosomal RNA synthesis which is highly active in oogenesis (Pfaff and Anderer, 1988). There also seems to be some staining of mitochondria which could point to some yet undiscovered function of CK2.

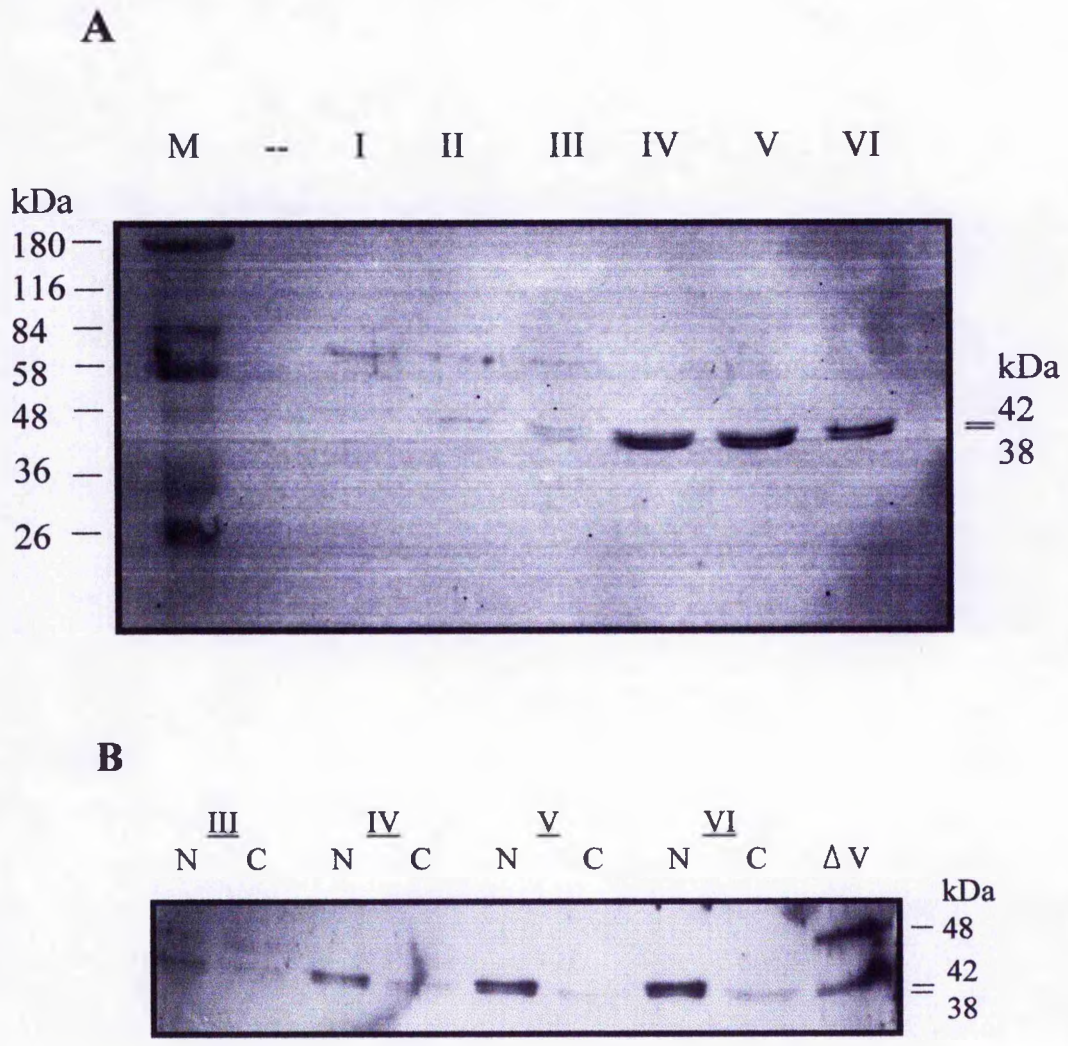



**Figure 16: Levels of CK2α during oogenesis**: A) Anti- CK2α immunoblot of oocyte extracts from stages I-VI. Each track contains approximately two oocytes of extracted protein. This is a DAB reaction producing a stain of immuno reactive protein. The markers are prestained and are not reacting with the antibodies or with the DAB reagents. The first track is protein markers of 180, 116, 84, 58, 48, 36 and 26 kDa. The two α-subunits are seen at 42 and 38kDa. There may be under-representation of the reactivity from stage III oocytes in this particular blot. B) Anti-CK2α immunoblot of nuclear (N) and cytoplasmic (C) protein extracts from stages III-VI. Each nuclear track contains approximately 10 nuclei worth of extracted protein and each cytoplasmic track contains approximately 2 cytoplasms. The fusion protein GST-HDΔV is present at 48 and 40 kDa as a positive control. It reacts with anti-CK2α because the GST portion of the antigen is present in both.

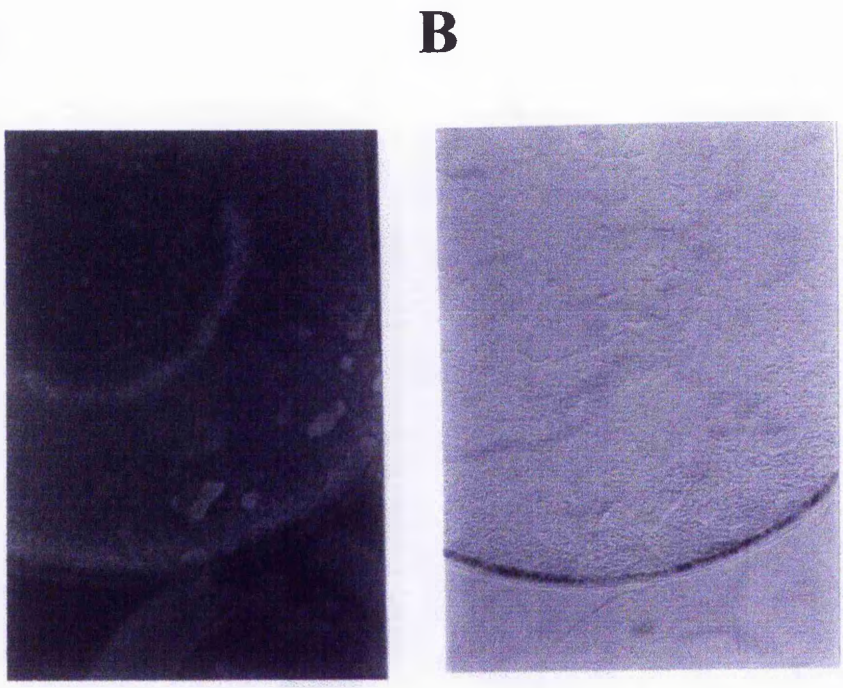
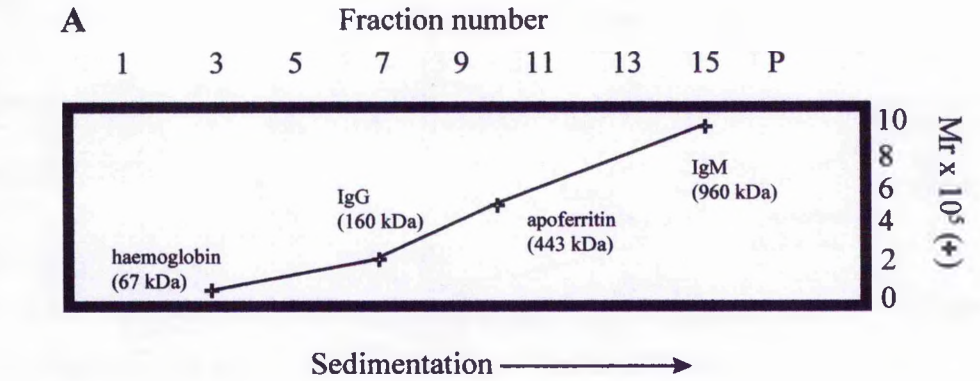


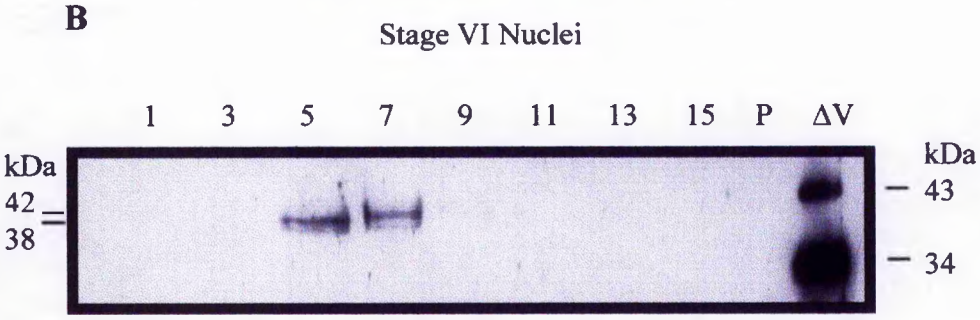

**Figure 17: Immunostaining of sectioned ovary:** A) Image of stage III oocytes stained with anti-CK2α using a fluorescence secondary antibody at 1/100 dilution. B) Phase partner to section in A. The nuclear envelope, mitochondria and nucleoli are positive for CK2α. Magnification x392.

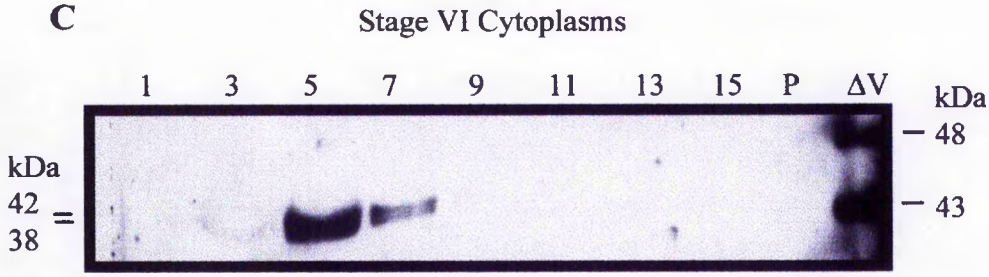
*3.4.2 The CK2 particle during oogenesis*: In order to study the characteristics of CK2 in oocytes, it was necessary to study the tetrameric complex itself. Since the clones expressing CK2 did not have any enzymatic activity, another way was needed to help investigate the activity of CK2. The size and activity of the native form of CK2 had to be studied directly. This was done by running oocyte extracts on glycerol gradient to determine the actual particle size and to check coincidence of immunoreactivity and enzyme activity. One question to answer was whether CK2α existed as a free subunit, part of a tetrameric complex, or part of any larger complexes of proteins.

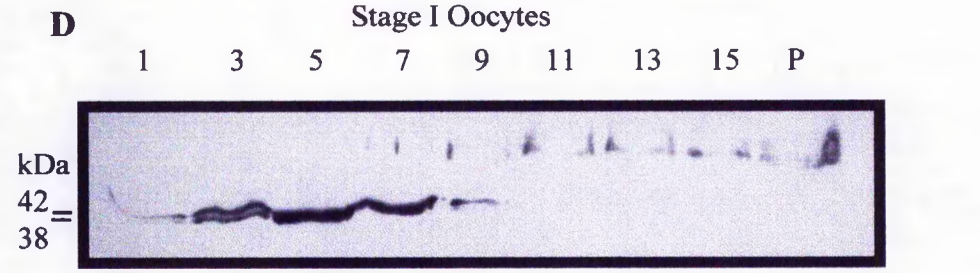
Sample of whole oocytes, nuclei and cytoplasms were solubilized using the same method as described above (see Methods, section 2.9). The gradients were loaded with oocyte extracts usually treated with Freon to remove the lipid which could interfere with the migration of particles. The samples were run at 36,000 rpm for 16 hrs in a 10-30% glycerol gradient (see Methods, section 2.10) These gradients were then fractionated into sixteen 150 µl samples, plus an insoluble pellet fraction. The fractions were acetone precipitated, raised in 10µl of 8M urea and 10µl of SDS-PAGE buffer, and run on a protein gel (see Methods, section 2.6). The gel was then Western blotted (see Methods, section 2.7). Marker sizes were obtained by running a gradient containing marker proteins and protein complexes in parallel with the samples which was fractionated, separated by SDS-PAGE and stained to identify the sedimentation rates.

Gradients of different oocyte extracts were run in an attempt to locate the CK2α associated particle, and to determine if this particle occurred at the same size throughout oogenesis. Stage I, III, and VI oocytes were blotted after the gradient was fractionated to identify the particle size. The fractions were precipitated with four volumes of acetone in order to concentrate the proteins and the precipitate was run on SDS gels. These were transferred to nitrocellulose paper and immunoblotted with anti-CK2α. Stage VI oocytes were separated into cytoplasmic and nuclear fractions to see whether CK2 was associated with different sized particles in the cytoplasms compared to the nuclei. Figure 18 shows that the particle size appears to be constant throughout oogenesis. Fraction 3, 5 and 7 seem to contain most of the CK2α subunit, with a peak in fraction 5. These fractions from the gradients correspond to a particle of approximate size of 130 kDa, which is the predicted size of CK2 in its tetrameric


A
Fraction number
1 3 5 7 9 11 13 15 P
haemoglobin
(67 kDa)
IgG
(160 kDa)
apoferritin
(443 kDa)
IgM
(960 kDa)
10
8
6
4
2
0
Mr x 10⁵ (+)
Sedimentation


B
Stage VI Nuclei
1 3 5 7 9 11 13 15 P ΔV
kDa
42
38
kDa
43
34


C
Stage VI Cytoplasms
1 3 5 7 9 11 13 15 P ΔV
kDa
42
38
kDa
48
43


D
Stage I Oocytes
1 3 5 7 9 11 13 15 P
kDa
42
38

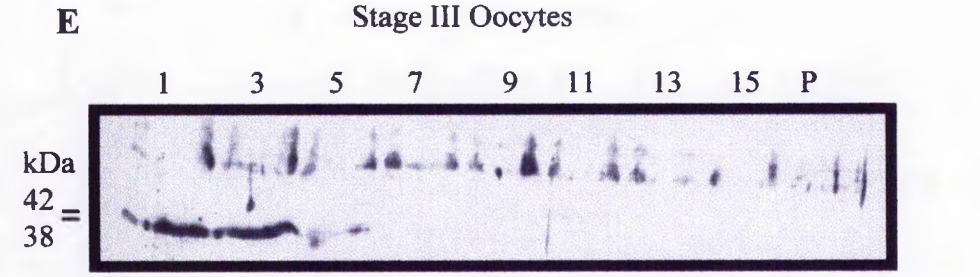

E
Stage III Oocytes
1 3 5 7 9 11 13 15 P
kDa
42
38

**Figure 18: Sedimentation analysis of protein complexes that contain CK2α protein: A)** Protein size markers **B)** Immunoblot with anti-CK2α of soluble extracts from 100 stage VI nuclei recovered from alternative fraction of a glycerol gradient. The material pelleted in the gradient (P) and fusion protein GST-HDΔV are included in the blot. **C)** As in A), but from a gradient used to separated the Freon-treated extract of 100 stage VI cytoplasms. **D)** As in A), but from a gradient used to separate the Freon-treated extract of 50 stage I oocytes. GST-HDΔV was not added as a marker. **E)** As in C), but from a gradient used to separate the Freon-treated extracts of 50 stage III oocytes.

form. The figure 18 E shows higher cross reacting bands across the gradient, but this was a reprobed western blot which was not completely stripped of a previous antibody reaction. Also in figure 18E, the signal for CK2α appears in lower fraction numbers compared to the other gradients, this gradient was spun slower (30,000 rpm) than those shown in B-D.

*3.4.3 CK2 activity in nuclear gradient fractions*: In order to confirm that the particle sizes that were being identified by the immunoblots actually represented the active protein kinase, a suitable protein substrate GST-HDΔV (see Chapter 4) was added to the fractionated gradients along with [$^{32}$P]γ-ATP, to identify which fractions had the ability to phosphorylate this substrate.

Gradient fractions were incubated with 1μl of [$^{32}$P]γ-ATP and 2μl of GST-HDΔV for one hour at room temperature. The samples were then precipitated with acetone to concentrate the phosphorylated protein, and to remove un-incorporated counts. The precipitated protein was raised in 8M urea and SDS-PAGE buffer and run on a protein gel, stained with Coomassie blue and destained to resolved the protein bands (see Methods, section 2.6). The gel was dried down onto 3M paper using a vacuum dryer and placed with X-ray film to visualised the radioactivity.

In figure 19 it can be seen that indeed GST-HDΔV is phosphorylated in fraction 5 and 7 which represents the highest levels of CK2α immunoblotting. Other kinase activities are represented, as can be deduced from the strong phosphorylation of endogenous nuclear proteins. The most prominent phosphorylation is apparent in fraction 9; this was mainly phosphorylation of larger proteins. This is to be expected since the nucleus contains many kinases, but the fact that they do not phosphorylate GST-HDΔV is consistent with the observation that CK2 sites are the most abundant sites occurring on the GST-HDΔV subclone of HDACm (see Chapter 4), and phosphorylation of this fusion protein is likely to be by CK2.

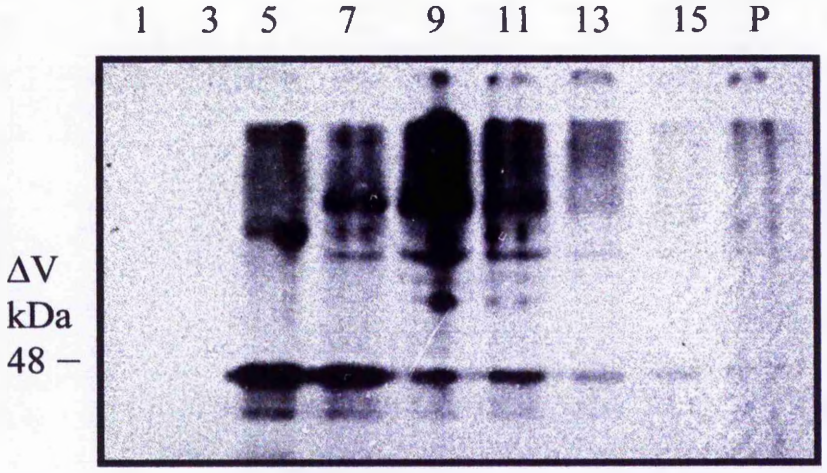



**Figure 19: Protein kinase activity in gradient fractions of stage VI nuclei**: Activity gel of 100 stage VI nuclei separated on a glycerol gradient. GST-HDΔV is added as the substrate and [$^{32}$P]γ-ATP added as the label.

This phosphorylation was quantified and graphed to give an accurate pictorial representation of the kinase activity (fig. 20).

The dried protein gel that was used to make the autoradiograph was scanned to measure the total number of incorporated counts. The activity in each band and track was measured using an instant imager (see Methods, section 2.15). Each track

was measured both for the GST-HDΔV radioactive band and for the endogenous protein activity in the highest molecular weight block.

In figure 20, the X axis represents the gradient fractions and the Y axis represents the total number of counts found in each. The blue line is a measurement of phosphorylation of GST-HDΔV. The blue line records the highest level of counts in fraction 5 and 7, which corresponds to particles at 130 kDa, the expected size of the CK2 tetramer. The red line represents the total number of counts of the higher molecular weight proteins found in each fraction; these are endogenous nuclear proteins. The highest activity is in fraction 9, which probably represents some other active nuclear kinase. The broken black line represents the size markers; the X axis represents the fraction number and the YY axis the molecular weight. The number of total counts as estimated for all the GST-HDΔV fractions and also for the larger nuclear proteins. The graph (fig. 20) shows that the greatest phosphorylation of GST-HDΔV was in 5 and 7, while the greatest activity phosphorylating endogenous proteins was in fractions 9 and 11. As stated earlier, this is understandable, because the nucleus contains many kinases; the crucial point here is that the highest number of counts measured for GST-HDΔV correspond to the immunoblotted CK2α fractions..

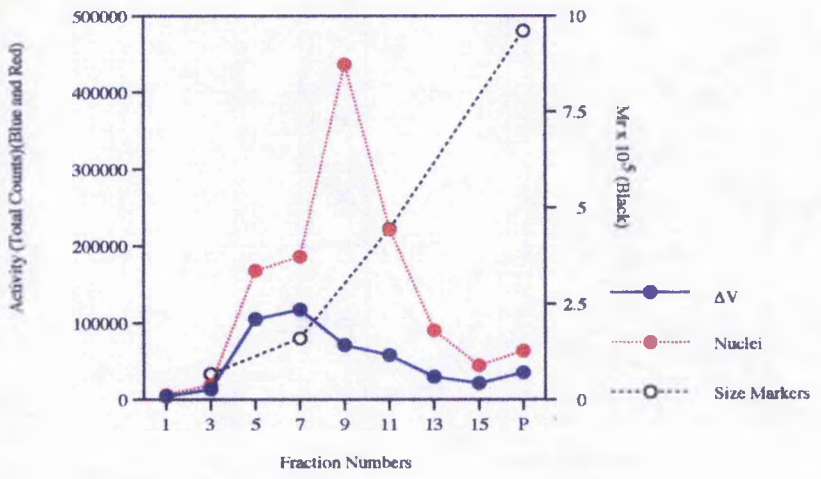



**Figure 20: Graph of gradient for stage VI nuclear activity**: Activity profile of soluble extracts from 100 stage VI nuclei using GST-HDΔV as a substrate (blue circles) and endogenous nuclear proteins as a substrate (red circles). Peak sedimentation positions of molecular mass marker (Mr) are shown (black circles).

## 3.5 Activity Study of CK2 with Various Substrates, Inhibitors and Buffers

The activity studies discussed in this section contained many different substrates and inhibitors but the reaction were generally carried out using the same procedure. A reaction mixture was made which contained 1μl of either nuclei or kinase extract, 1 μl of 10x kinase buffer, 1μl of [$^{32}$P]γ-ATP (0.03 mM), 2μl of a substrate (GST-HDΔV, α-casein, or Y-box protein), 1μl of an inhibitor (varying concentrations) and 4μl of $dH_2O$. These samples were left to incubate at room temperature between 30 minutes and an hour. 10μl of SDS-PAGE buffer was added to these samples which were then run on a protein gel. The gels were stained, destained, dried on to 3M paper, and set up for autoradiography (see Methods, section 2.6).

*3.5.1 Heparin inhibition*: Heparin is one of the most widely studied inhibitors of CK2 (Hathaway *et al.*, 1980; Taylor *et al.*, 1987; Allende and Allende, 1995). It

works by binding to the substrate binding site of CK2, thus preventing the enzyme from phosphorylating other substrates. Heparin is an efficient inhibitor of CK2 activity, as can be seen from figure 21. The equivalent of one nucleus, as the source of CK2, is added to the reaction, as well as the substrate GST-HDΔV at the final concentration of .04μg/μl. Heparin is effective at 100μg/ml concentrations, inhibiting almost all CK2 activity.

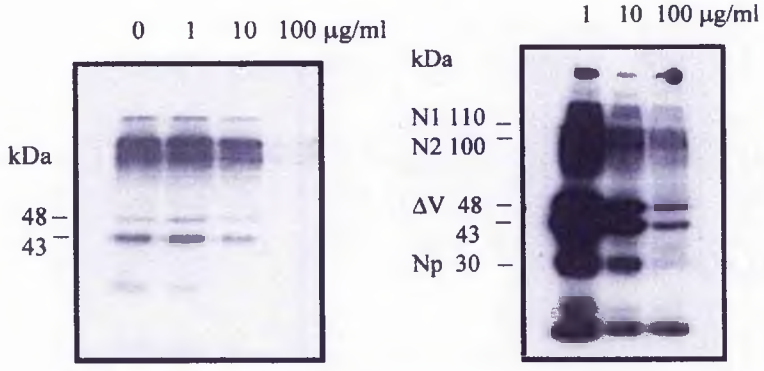



**Figure 21: Autoradiographs showing inhibition of nuclear CK2 activity by heparin**: Both panels represent phosphorylation of GST-HDΔV with stage VI nuclei after the addition of increasing amounts of the inhibitor heparin. The GST-HDΔV samples are marked as 48 and 43 kDa and the other most predominant proteins to be phosphorylated are N1, N2 and Nucleoplasmin at 110, 100 and 30 kDa.

*3.5.2 DRB inhibition*: Although the inhibitor DRB has not been used for as long as heparin has, as an inhibitor of CK2 activity, its use as an inhibitor is well documented (Zandomeni *et al.*, 1984; Vancurova *et al.*, 1995; and Egyhazi *et al.*, 1998). It works differently from heparin in that it binds to the ATP binding site of CK2, thus inhibiting its ability to bind ATP. In order to help define the activity of CK2, a DRB titration was carried out using nuclei and heparin-bound fraction as the sources of protein kinase activity to phosphorylate the substrate GST-HDΔV. This was done in the presence of [$^{32}$P]γ-ATP as a label. A sufficient concentration of DRB

in inhibiting phosphorylation by nuclear CK2 seem to be around 50μM and, when using extracted CK2, a somewhat higher concentration of 150μM seemed to be required (fig. 22).

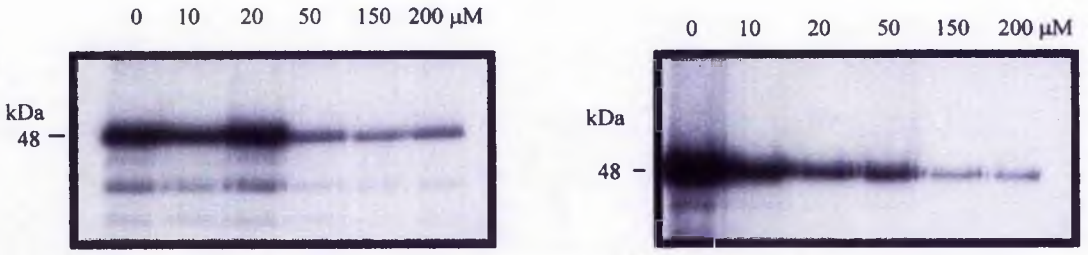



**Figure 22: Autoradiographs showing inhibition of CK2 activity by DRB**: The two panels represent the titration of the inhibitor DRB with stage VI nuclei (left) and extracted kinase activity (right) using GST-HDΔV as the substrate and [$^{32}$P]γ-ATP as the label. The figures show increasing concentrations of DRB and its effect on labeling of GST-HDΔV substrate.

*3.5.3 Quercetin inhibition*: Quercetin, like DRB, also inhibits CK2 by blocking the ATP-binding site. Its use as an inhibitor has also been well documented, and like heparin, seems to be very specific towards CK2 (Cochet *et al.*, 1982; Braddock *et al.*, 1994). A titration of quercetin was performed using the same technique as that used in the DRB titration. Stage VI nuclei and heparin-bound fraction were used as the sources of CK2 which was incubated in the presence of GST-HDΔV and [$^{32}$P]γ-ATP. As can been seen from figure 23, quercetin was very effective against stage VI nuclei with effective inhibition at levels as low as .06μM. Against the extracted CK2 it was also very effective, but at a slightly higher concentration of 0.3μM.

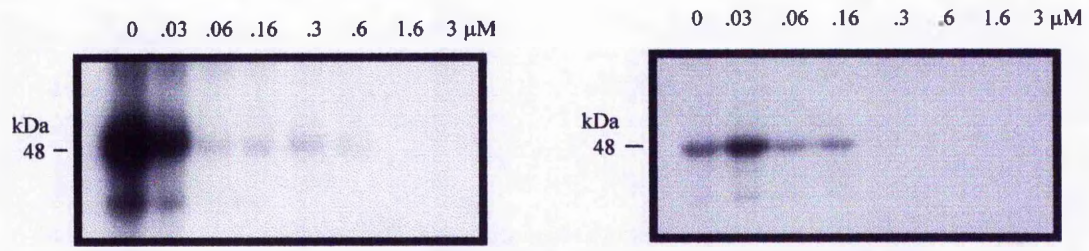



**Figure 23: Autoradiographs showing inhibition of CK2 activity by quercetin**: The two panels are the titration of the inhibitor quercetin with stage VI nuclei (left) and extracted kinase (right) using GST-HDΔV as the substrate and [$^{32}$P]γ-ATP as the label. The figures show increasing concentrations of quercetin and its effect on labeling of GST-HDΔV substrate.

*3.5.4 Rutin inhibition*: Rutin is an inactive analog of quercetin, thus it was used as a negative control (Cochet *et al.*, 1982; Braddock *et al.*, 1994). The titrations were repeated using the same procedure as that for the quercetin titrations. Stage VI nuclei and heparin-bound fraction were again used as the sources of kinase activity. The substrate for this reaction was GST-HDΔV, and [$^{32}$P]γ-ATP was used as the label. As can be seen from figure 24, there was no inhibition using the same concentrations used for rutin as used for quercetin when testing the stage VI kinase activity. Some inhibition was seen with extracted CK2, but it was not nearly as

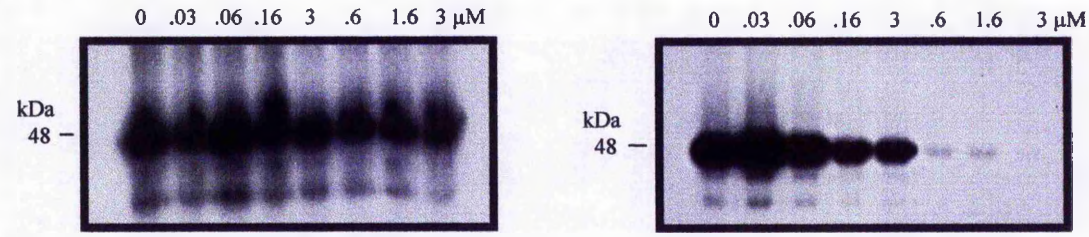



**Figure 24: Autoradiograph showing inhibition of CK2 activity by rutin**: The two panels represent the titration of the rutin with stage VI nuclei (left), and extracted kinase (right), using GST-HDΔV as the substrate and [$^{32}$P]γ-ATP as the source of label. Rutin concentrations increases left to right.

sensitive to rutin as with quercetin. The sudden decrease in activity at 0.6 μM was probably due to non-specific inhibition at this relatively high concentration.

*3.5.5 Varying buffer conditions*: CK2 activity is reported to be sensitive to concentrations of certain metal ions and salts (Gatica *et al.*, 1993; Allende and Allende, 1995). $MgCl_2$, specifically $Mg^{2+}$ ions, are a necessary cofactor for phosphorylation activity of CK2. To help define the activity of CK2, varying salt concentrations were added to a phosphorylation reaction to see how this would affect the activity of the kinase. In figure 25 NaCl and KCl were added to the reaction mixture which contained nuclei from stage VI as the source of the kinase activity, GST-HDΔV as the substrate and [$^{32}$P]γ-ATP as the source of label. The salts were added in increasing concentrations, and as can be seen from figure 25, NaCl, even at low concentrations, had a negative effect on kinase activity. KCl at low concentrations of 100 mM had little effect, but as soon as the concentration was raised to 200mM and especially at 400mM, the kinase activity dropped steeply below its normal levels. The $MgCl_2$ level is also very important to activity. The addition of 2mM EDTA, which chelates divalent cations such as $Mg^{2+}$ and $Ca^{2+}$, had an inhibitory effect on the efficiency of phosphorylation, and the addition of moderate amount of $MgCl_2$ (20mM final) improved the phosphorylation activity greatly compared to the lower $MgCl_2$ (10mM final) reaction and the EDTA reaction (2mM).

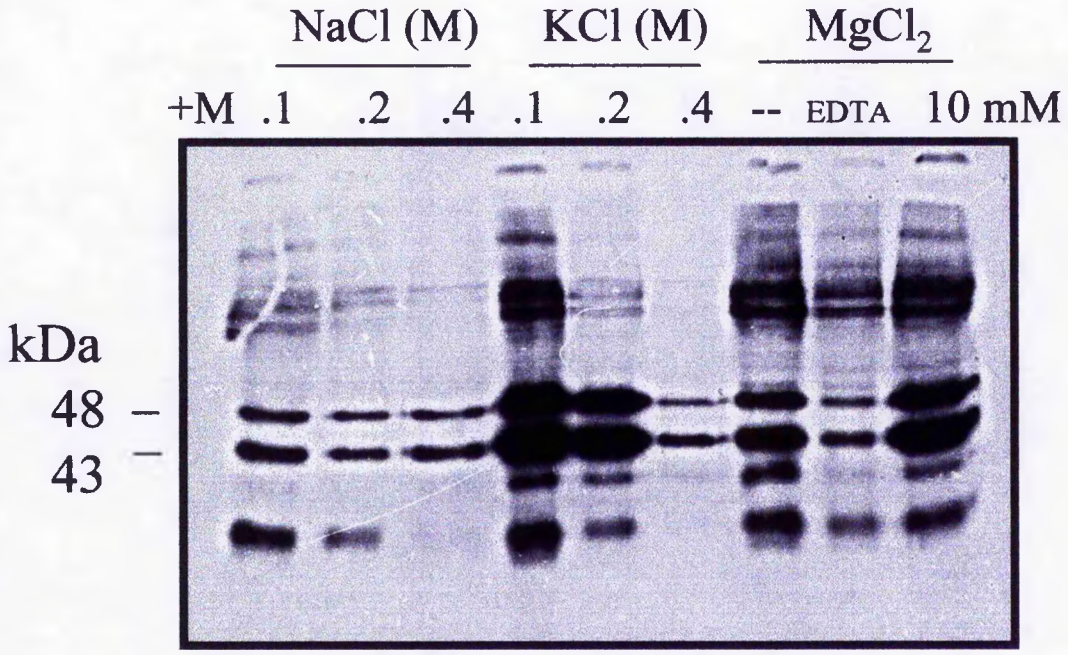



**Figure 25: Autoradiograph showing phosphorylation of GST-HDΔV with CK2 from Stage VI nuclei with varying buffer conditions**: The first three tracks illustrates the effects of adding increasing amounts of NaCl from 100mM-400mM. The next three tracks illustrates the addition of increasing amounts of KCl. The final three tracks shows the effect of: no addition of $MgCl_2$, the removal of divalent cations with the addition of 2mM EDTA, and the effect of the addition of an extra 10mM $MgCl_2$ to the reaction mixture. 1x kinase buffer is 100mM KCl, 10mM $MgCl_2$, 20mM Tris and 1 mM DTT.

*3.5.6 Polypeptide, poly(EY), inhibition of phosphorylation*: Poly(EY) was used as a competitor inhibitor for CK2 activity due to the fact that it contains the acidic residues glutamic acid intermixed with tyrosine at a ratio of 4 to 1. This compound binds very efficiently to the substrate binding site of CK2. CK2 recognises S/T XX D/E sequences, normally in an extended acidic region in potential substrates, so poly(EY) added in high concentration competes out other substrates containing those sites. Figure 26 shows that the substrates GST-HDΔV, p60/56 and α-casein are not phosphorylated after the addition of poly(EY); this indicates that all these substrates are phosphorylated by a kinase which recognises the aforementioned acidic substrate

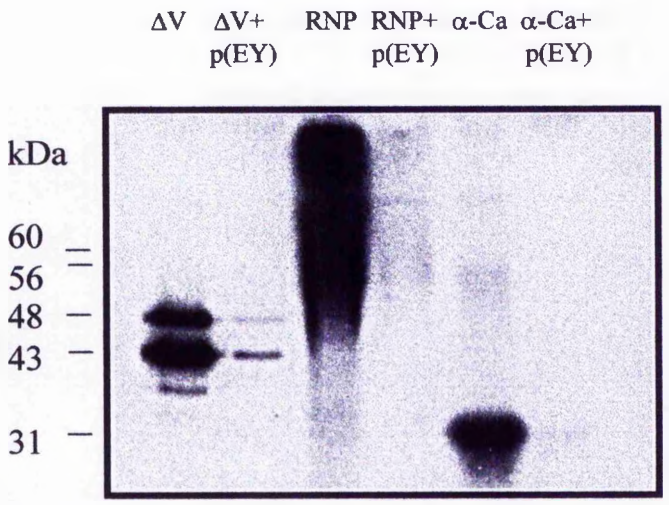



**Figure 26: Autoradiograph showing inhibition of phosphorylation of CK2 substrates by the synthetic polypeptide poly(glutamic acid: tyrosine)**: This first track is the phosphorylation of GST-HDΔV. In the second track phosphorylation after the addition of poly(EY) to 1μg/ml. The third track is the phosphorylation of purified RNP proteins. The fourth track is as 3, but after the addition of poly(EY). The fifth track shows phosphorylation of α-casein (α-Ca). The sixth track is after the addition of poly(EY).

binding motif. This is again evidence that CK2 is the major protein kinase which phosphorylates these substrates.

In order to learn more about the binding ability of CK2, another potential inhibitor was added to a phosphorylation reaction, but this substrate was composed of multiple glutamic acid residues, poly(E). The binding of CK2 is dependent on specifically placed acidic residues, but how important other residues, specifically those providing a hydroxyl group, are for binding of CK2 needed to be evaluated. The poly(E) competitor was added to a phosphorylation reaction with stage VI nuclei and GST-HDΔV used as the substrate (fig. 27). The ability of poly(E) to inhibit phosphorylation is very poor compared to the ability of poly(EY), which illustrates the point that not only are the acidic residues necessary for binding to CK2 but that neighbouring hydroxylated side chains are also important. In the form of serine or

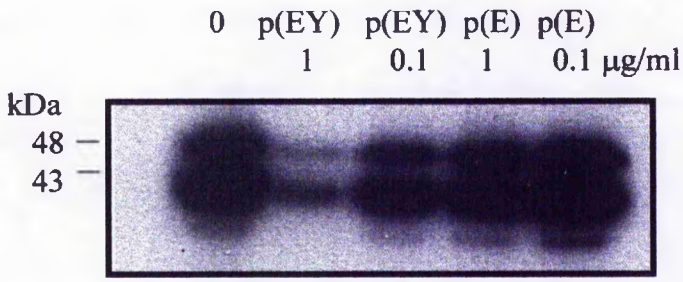



**Figure 27: Autoradiograph showing a comparison of inhibition of CK2 phosphorylation by poly (glutamic acid: tyrosine) and poly (glutamic acid)**: The first track is phosphorylation of GST-HDΔV. The second is phosphorylation of GST-HDΔV with 1 μg/ml of poly(EY) added. The third track is phosphorylation of GST-HDΔV with 0.1 μg/ml of poly(EY) added. The fourth track is phosphorylation of GST-HDΔV with 1 μg/ml of poly(E) added. The fifth track is phosphorylation of GST-HDΔV with 0.1 μg/ml of poly(E) added.

threonine they may be phosphorylated by CK2, in the form of tyrosine some binding affinity may be provided but modification is not achieved.

*3.5.7 Inhibition of CK2 phosphorylation by anti-CK2α*: The last potential inhibitor of CK2 activity to be tested was the antibody against the 2A clone. If the sites recognised by the antibody were part of the substrate binding site, or the ATP binding site, then it may have an effect on the activity of CK2. Figure 28 shows a phosphorylation of GST-HDΔV using stage VI nuclei after the addition of increasing dilutions of anti-CK2α serum. The gel shows that at 1/10 dilution, there is no change in phosphorylation levels. This result may be due to the fact that there is a very high concentration of serum which for some reason gave an anomalous result. The second dilution of 1/100 showed significant inhibition of phosphorylation of GST-HDΔV. A similar result was observed for the next dilution of 1/1000. A control antibody was used in the same manner and there was a less significant loss of phosphorylation using the control. This seems to show that the antibody can reduce the activity of the native kinase. Although it must be stated that even though an antibody does bind to a

particular enzyme, it does not necessarily mean that the antibody can affect its activity. This antibody seems to be able to effect the phosphorylation activity of the CK2 only to a limited extent and was judged to be of little use for further inhibition studies.

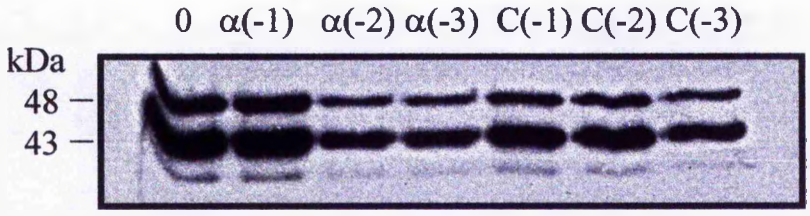



**Figure 28: Autoradiograph showing the effect of preincubation with immune and non-immune serum on phosphorylation by nuclear CK2**: The gel shows the effect of increasing dilutions 1/10 (-1), 1/100 (-2),and 1/1000 (-3) of anti-CK2α (α) and of a control antibody (C) on the phosphorylation of GST-HDΔV by CK2.

### 3.6 Summary

The protein kinase CK2 is expressed in increasing amounts during oogenesis, reaching the highest levels in large oocytes: stage IV, V and VI. Although the amounts of CK2 are high in both the cytoplasm and nucleus, it seems to exist in a higher concentration in the nucleus. In tissue sections, it is seen to be most widely associated with the nuclear envelope and nucleoli. Sedimentation analysis of the native enzyme shows that it exists mostly as a 130 kDa particle thoughout oogenesis, and that the phosphorylation activity for known CK2 substrates corresponds with this particle.

Analysis of its activity shows that the inhibitors DRB, quercetin and heparin all have the ability to inhibit both the nuclear and partially purified form of CK2. Varying salt and $Mg^{2+}$ ion concentrations significantly affect the activity of the kinase; this is an observation which has already been reported for CK2 from other

sources (Gatica *et al.*, 1993; Allende and Allende, 1995). Specific substrate competitors such as the synthetic polypeptide poly(EY) show efficient reduction of phosphorylation levels of test substrates while control, poly(E), shows little effect. All of the tests carried out and described in this chapter indicate that the phosphorylation activity being studied is indeed due to the protein kinase CK2. Since stage VI nuclei are a good source of the native tetrameric complex, nuclear extract and CK2 partially purified from nuclei provide a convenient source of enzyme activity for further experimentation.

# Chapter 4: Results

# Expression and Regulation of Histone Deacetylase

## 4.0 Aim

A histone deacetylase (HD) expressed in *Xenopus* oocytes, HDACm (Sommerville *et al.*, 1998), contains several putative CK2 phosphorylation sites, suggesting that CK2 could be a regulator of HD enzyme activity, or some other functions such as, nuclear import of HD. This chapter explores the expression of HDACm in *Xenopus* oocytes, nuclei and cytoplasms in order to understand how its activities may relate to phosphorylation by CK2. The effects of phosphorylation on HD activity and the effects of phosphorylation on the kinetics of nuclear transport of HDACm are studied. The issue of how phosphorylation mediates the interaction between the nuclear localisation signal (NLS) of HDACm and the nuclear shuttling protein α-importin is also studied to try and discover what specific function CK2 could have on the nuclear import mechanism.

## 4.1 Cloning of HDACm Gene

*4.1.1 Xenopus AB21*: The *Xenopus* form of histone deacetylase was first cloned by a student named Scott Lyons working in John Sommerville's lab in 1994. The clone named AB21 (accession number X78454) was recognized as a candidate homologue of the yeast global transcription regulator RPD3 (Vidal and Gaber, 1991). This was followed by the later cloning of a mammalian histone deacetylase (Taunton *et al.*, 1996) which made it evident that both RPD3 and AB21 were members of this same enzyme family.

```
                                                        GCGGAAGGAAA   -11
ATGGCGCTGACTCTAGGAACAAAGAAGAAAGTGTGCTACTACTATGATGGTGATGTTGGA    60
 M  A  L  T  L  G  T  K  K  K  V  C  Y  Y  Y  D  G  D  V  G
AATTATTATTATGGTCAAGGGCATCCCATGAAACCTCATAGAATTCGCATGACACACAAC   120
 N  Y  Y  Y  G  Q  G  H  P  M  K  P  H  R  I  R  M  T  H  N
CTGCTGCTCAACTATGGACTTTACCGAAAAATGGAAATCTTTAGGCCCCACAAAGCCAGC   180
 L  L  L  N  Y  G  L  Y  R  K  M  E  I  F  R  P  H  K  A  S
GCCGAGGATATGACAAAGTATCACAGTGATGATTATATCAAATTCCTGCGCTCCATACGA   240
 A  E  D  M  T  K  Y  H  S  D  D  Y  I  K  F  L  R  S  I  R
CCAGACAATATGTCCGAATACAGTAAACAGATGCAGAGATTTAATGTTGGTGAGGACTGT   300
 P  D  N  M  S  E  Y  S  K  Q  M  Q  R  F  N  V  G  E  D  C
CCTGTGTTTGATGGCCTATTTGAGTTCTGCCAGCTCTCTGCAGGGGGTTCTGTAGCAAGT   360
 P  V  F  D  G  L  F  E  F  C  Q  L  S  A  G  G  S  V  A  S
GCTGTTAAACTAAACAAACAGCAGACTGACATTTCAGTGAACTGGTCTGGTGGCCTTCAT   420
 A  V  K  L  N  K  Q  Q  T  D  I  S  V  N  W  S  G  G  L  H
CATGCAAAGAAATCTGAGGCATCTGGTTTTTGTTATGTCAACGATATTGTCCTTGCCATC   480
 H  A  K  K  S  E  A  S  G  F  C  Y  V  N  D  I  V  L  A  I
CTGGAACTACTAAAGTATCACCAGAGAGTTGTGTATATTGATATAGACATTCACCACGGT   540
 L  E  L  L  K  Y  H  Q  R  V  V  Y  I  D  I  D  I  H  H  G
GATGGTGTTGAGGAGGCATTTTACACAACCGATAGGGTTATGACTGTGTCCTTCCATAAG   600
 D  G  V  E  E  A  F  Y  T  T  D  R  V  M  T  V  S  F  H  K
TATGGAGAGTATTTTCCTGGAACTGGAGATCTGAGAGATATTGGTGCAGGGAAAGGCAAA   660
 Y  G  E  Y  F  P  G  T  G  D  L  R  D  I  G  A  G  K  G  K
TACTATGCTGTAAATTATGCCTTACGGGATGGGATTGACGATGAGTCCTATGAAGCAATT   720
 Y  Y  A  V  N  Y  A  L  R  D  G  I  D  D  E  S  Y  E  A  I
TTTAAACCAGTAATGTCCAAAGTTATGGAAATGTTTCAGCCCAGTGCAGTGGTCTTACAG   780
 F  K  P  V  M  S  K  V  M  E  M  F  Q  P  S  A  V  V  L  Q
TGCGGAGCAGATTCATTATCTGGGGATAGACTGGGATGCTTCAATTTGACCATTAAGGGA   840
 C  G  A  D  S  L  S  G  D  R  L  G  C  F  N  L  T  I  K  G
CATGCGAAGTGTGTGGAGTTTATAAAGACCTTTAACTTGCCACTGTTGATGTTAGGAGGT   900
 H  A  K  C  V  E  F  I  K  T  F  N  L  P  L  L  M  L  G  G
GGAGGTTACACTATCCGGAATGTGGCTCGTTGCTGGACATATGAAACAGCTGTGGCTCTG   960
 G  G  Y  T  I  R  N  V  A  R  C  W  T  Y  E  T  A  V  A  L
GACTCTGAGATCCCCAATGAGCTTCCATATAATGATTATTTTGAATATTTTGGTCCGGAC  1020
 D  S  E  I  P  N  E  L  P  Y  N  D  Y  F  E  Y  F  G  P  D
TTCAAGCTTCACATCAGCCCATCCAACATGACTAATCAGAACACTAATGAATATCTGGAG  1080
 F  K  L  H  I  S  P  S  N  M  T  N  Q  N  T  N  E  Y  L  E
AAAATTAAGCAGCGCCTCTTTGAGAACTTGCGCATGCTCCCCCATGCTCCTGGAGTTCAG  1140
 K  I  K  Q  R  L  F  E  N  L  R  M  L  P  H  A  P  G  V  Q
ATGCAAGCCGTTGCAGAGGACTCCATACACGATGACAGTGGTGAAGAAGATGAAGATGAT  1200
 M  Q  A  V  A  E  D  S  I  H  D  D  S  G  E  E  D  E  D  D
CCCGACAAGCGTATTTCAATTCGGTCATCAGATAAAAGGATTGCCTGTGATGAGGAGTTC  1260
 P  D  K  R  I  S  I  R  S  S  D  K  R  I  A  C  D  E  E  F
TCAGATTCTGAGGATGAAGGGGAGGGAGGTCGCAAAAACGTGGCCAATTTCAAAAAAGTA  1320
 S  D  S  E  D  E  G  E  G  G  R  K  N  V  A  N  F  K  K  V
AAACGGGTTAAAACTGAAGAGGAAAAGGAAGGAGAGGACAAGAAAGATGTTAAAGAAGAG  1380
 K  R  V  K  T  E  E  E  K  E  G  E  D  K  K  D  V  K  E  E
GAGAAAGCTAAAGATGAGAAGACGGATAGCAAACGGGTAAAAGAAGAGACCAAATCAGTC  1440
 E  K  A  K  D  E  K  T  D  S  K  R  V  K  E  E  T  K  S  V
TGATCCTTCAACTATGGGGAGAAAATCCGAAGACCAAACTAATTCTCATGGTTTTATATT  1500
TTGTATATGCCCTGTACAGAGCCCTACTATGAAATATAAGTCCACACATTCTAAATTATT  1560
TCTGTCCCACTGGTTGAGGGGGGGTGAAGTGGTCGCTGTAGTGGATTAAGCTTCACATCT  1620
GTTACCTTTTTTTAAGATTCACATCTGTTACCTTTTTACCAGATGTTTCCAGCTCTTTGG  1680
CTTTTTTTTTTTTTTTGACCAAAAACTTTCCATGTTTTCCTGTGCCTCTGTAATCTTCGG  1740
TGGTGCAATGCATTACGGATTTATTTCCCTGCTCCCTTCTATACACACTTTGCTGTCAGA  1800
CTACAGACTTTTGCTACAGTACATGAAATATGTACACTTATGCTCAGGATCAGGCATATG  1860
TACACTTATGCTCAGGATCAGGCAGTGAGAAGGAGGTGGGTTCCAGCTGTCTTCCAAATG  1920
AATTTGAGAGGGTTACCTTGAGGGATGGAAGGGGGAAGCTGAAGCTCCTCTTAAACTAAA  1980
CTATTCAGGGATTCCCTGTTCACTTAATGCTGCTAACCCTCCTCCAGATTAGTTCATGAA  2040
GCAGATTTTTAGATGTGTGGAAACCTGGTCCACAGTTACCTTATAATGGGGATTGTGGGG  2100
ATTTGCAATTTGGGTTTCTGCCTTTAATCTTAGTGGGTTGGAGAGTGTCTGGATTCATGG  2160
AGTGAAGAAAATGGAGAATTTTTATGTCTAATTTTTGTGATGGGAAATTTCTTTTTTTTT  2220
TTTTTTATGGTTGAGTTGTAGAAAAGCTTTGTAATAAAATCTGGTACTTATACAAAAAAA  2280
AAAAAAAAAAAAA                                                  2305
```

**Figure 29: AB21 cDNA Sequence**: Nucleotide and amino acid sequence of AB21. Potential CK2 phosphorylation sites are in bold and potential cleavage sites for trypsin are underlined. A putative bipartite NLS is in red italics.

*4.1.2 Subclones of AB21*: Subclones of AB21 were made by Michael Ladomery and consisted of glutathione fusions with varying lengths of the AB21 cDNA. These clones were thought to represent important sequences coding for functional domains of the native enzyme. The three most commonly used subclones were GST-HDΔV, ΔR and ΔR/ΔH, after the flanking restriction enzyme digestion sites. The GST-HDΔV subclone consisting of the tail domain, coding for the potential regulatory region of HDACm from an internal Pvu II site to the end of the clone. The GST-HDΔR subclone consists of most of the HDACm gene but missing 20 amino acids from the N-terminus to the internal Eco RI site. The GST-HDΔR/ΔH subclone represents the middle portion of the sequence, containing neither the N-terminus or C-terminal domain, after being cut with Eco RI and Hind III. This conserved region is thought to represent the enzyme core (Ladomery *et al.*, 1997a).

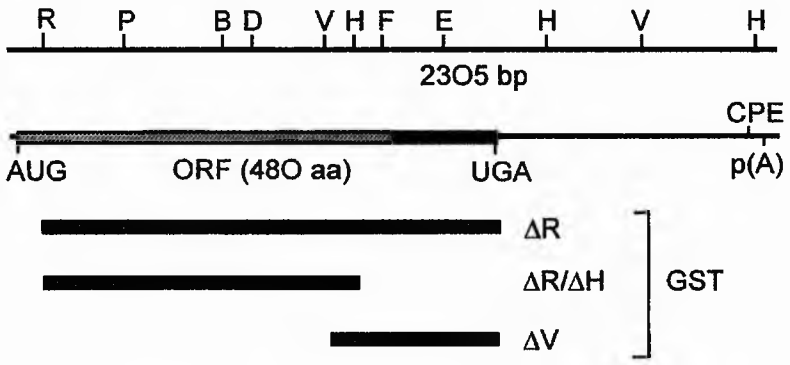



**Figure 30: HDACm subclones**: The three subclones of HDACm. ORF represents the open reading frame consisting of the sequence conserved between various histone deacetylases (gray) and the highly variable C-terminal region (black). The top line represents the approximate positions of existing restriction sites within the HDACm cDNA and within the subclones. R=Eco RI, P=Pst I, B=Bgl II, D=Dra I, V=Pvu II, H=Hind III, F=Fsp I, and E=Eae I.

*4.1.3 Antibodies against HDACm clones*: Antibodies against HDACm subclones (anti-ΔV, anti-ΔR, and anti-ΔR/ΔH) were produced by injection of the

glutathione fusion proteins into rabbits (see Methods, section 2.5 and 2.14). A fourth antibody (anti-Cpep) against a synthetic peptide which represents the terminal seventeen amino acids of HDACm was received from Dr. Brian Turner. These four antibodies all recognized the 57 kDa HDACm protein present in *Xenopus* oocytes extracts separated on protein gels.

### 4.2 Expression and Activity of HDACm in *Xenopus* Oocytes

*4.2.1 HDACm levels during oogenesis*: The levels of HDACm protein in oocyte extracts were measured by immunoblotting protein transferred to nitrocellulose paper from SDS-PAGE gels. Oocytes proteins were solubilized by bursting approximately 50 oocytes in homogeneization buffer (see Methods, section 2.9.3). Extracts were then spun at 10,000 rpm to separate the soluble fraction from the yolk and pigment pellet and the top lipid phase (see Methods, section 2.9.3). A sample of this soluble fraction was mixed with SDS-PAGE buffer and run on a protein gel (see Methods, section 2.6). Proteins from the gel were then transferred to nitrocellulose paper and reacted with the primary and secondary antibody (see Methods, section 2.7). Figure 31A shows that the levels of soluble HDACm increases steadily throughout oogenesis to peak in matured stage VI oocytes (stage VI treated with progesterone for 16 hrs). Immunoblots of nuclear and cytoplasmic levels (fig. 31B) show that there is a similar steady increase of HDACm in the nucleus as is seen in the whole cell extracts. The levels in the cytoplasm are low compared to that of the nucleus. Although the gel tracks contained material from 10 nuclei compared to 2 cytoplasms, the lack of signal seen in the cytoplasm tracks indicates a much lower level per-nucleus. This observation would tend to suggest that HDACm is transported efficiently into the nucleus.

*4.2.2 HDAC activity during oogenesis:* The activity of HDAC in the same extracts was assayed by the group of Dr. Brian Turner using an *in vitro* assay (see Methods, section 2.20). The deacetylase substrates were prepared using the same methodology stated in Sendra *et al.* (1988).

The activity of the HDAC from these same oocyte samples showed (fig. 31C) that the amount of activity correlated with levels of expressed HDACm protein. Furthermore the total amount of enzyme activity per embryo at cleavage, blastula and gastrula remained essentially the same as in the full-grown oocyte. However, the difference between the per cell amount of HDAC activity from oogenesis though early embryogenesis is worth attention. Due to the early embryonic cell divisions approximately a 10,000 fold higher level is present in oocytes compared with mid-blastula on a per cell basis. The higher levels of available enzyme activity in oocytes suggests that *in vivo* its regulation is efficient, because the lampbrush chromosomes of oocytes contain hyperacetylated histones (Sommerville *et al.,* 1993) and stored H4 remains di-acetylated during oogenesis (Almouzni *et al.,* 1994). Thus, it would seem that the bulk of the deacetylase activity is kept separated from its potential substrates, but by what means is unclear.

Past experiments on *Zea mays* histone deacetylase showed a sensitivity of substrate specificity to treatment with alkaline phosphatase (Brosch *et al.*, 1992). A dependence of activity on the phosphorylation state of the *Xenopus* enzyme was suggested by the effect of treating extracts with alkaline phosphatase. Oocyte and embryo extracts were treated with 50 units/ ml of alkaline phosphatase (Sigma, type III), adjusted to pH 8.3, and incubated for one hour at 22°C. Figure 32 shows that a

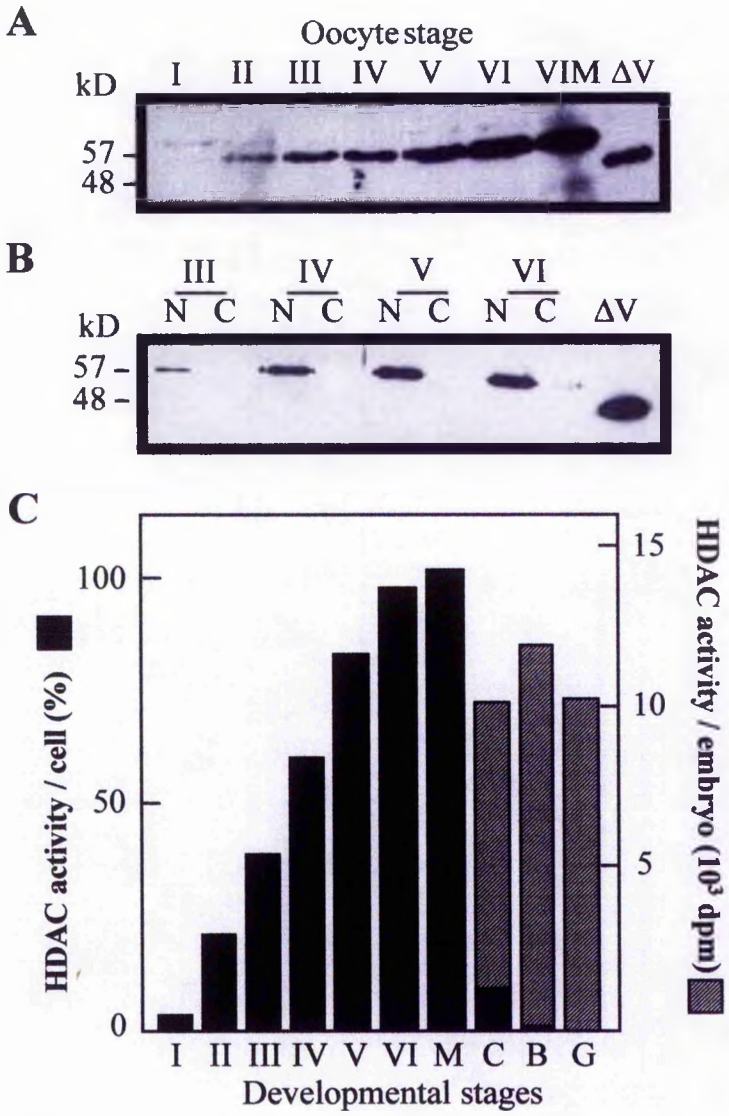



**Figure 31: Levels of HDACm Protein and HDAC Activity During Oogenesis**: A) Immunoblot of oocyte extracts from stage I-VIM (VIM=progesterone treated for 16 hrs.) using anti-Cpep. Two oocytes-worth of protein added to each track. 0.1μg of GST-HDΔV was added as positive control at 48kDa. B) Same as in (A) except whole oocytes range from stage III-VI and each are separated into nuclei (N) and cytoplasms (C). Nuclei tracks contain the equivalent of ten nuclei. C) Activity assay of histone deacetylase in oocyte stages I-VIM and embryos stages C (8-cells), B (mid-blastula) and G (gastrula). The activity is expressed as dpm of $^{3}$H-acetate released from histone by extracts of one oocyte or embryo (represented by the column's total height). The relative amount per cell is also indicated (%, black column height), which becomes smaller as cell divisions increase in number.

70-90% loss of HDAC activity was observed with this treatment in the in vitro assay compared to the activity without the phosphatase treatment. These assays were carried out on extracts supplied to the group of Dr. Brian Turner.

This dramatic drop in activity after incubation of extracts with alkaline phosphatase indicates the importance of phosphorylation to histone deacetylase activity. Which phosphorylation sites influence activity , and which protein kinase is responsible are not known.

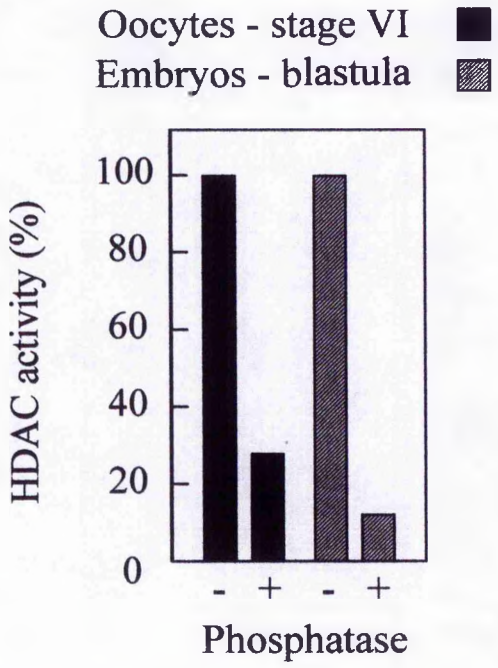



**Figure 32: Sensitivity of HDAC Activity to Phosphatase Treatment**: Extracts of oocytes (black column) or embryos (lined column) were incubated in the presence (+) or absence (-) of 2U/μl alkaline phosphatase.

### 4.3 Size of Protein Complexes with Histone Deacetylase Activity

The size of particles containing histone deacetylase activity were measured by rate-centrifugation of extracts, from *Xenopus* stage VI oocytes. Supernatants of oocyte extracts were layered onto 10-25% linear glycerol gradients and centrifuged until an IgM 19S marker reached the bottom of a gradient run in parallel with the

samples (see Methods, section 2.10). The gradients were then fractionated into 16 equal portions plus the pelleted material. These fractions were analysed for HDACm by immunoblotting and enzyme activity by the *in vitro* assay. Figure 33A shows that the activity in whole oocyte extracts, spun at 36,000 rpm for 16 hrs, peaks sharply at a position in the gradient corresponding to a particle size of ~360 kDa. This would indicate that the active form of histone deacetylase exists as part of a large protein complex of about 360 kDa. An immunoblot of proteins from the same gradient fractions (fig.33D) shows that the activity seen corresponds to a immunoreactive protein of 57 kDa which corresponds to HDACm. Gradient separation of material from nuclei isolated from stage VI oocytes shows a similar peak of activity at ~360 kDa (fig. 33B). Immunoblots of the same fractions again shows that the activity corresponds to the 57 kDa protein, which is most abundant in the nucleus than in the cytoplasm (fig. 33B and C). Pretreatment of stage VI oocytes with progesterone shows no change of HDAC activity levels or sedimentation rates.

Data indicates that HDACm exists as part of a protein complex and is contained mainly in the nucleus during the later stages of oogenesis. As was shown in Section 3.4.2., CK2 also accumulates in the nucleus becoming most abundant at the later stages of oogenesis. The protein complex containing CK2α and showing kinase activity is noticeably smaller than the protein complex containing histone deacetylase, and so there is no evidence for CK2 in the HDACm particle itself. Any modifications of HDACm by CK2 is likely to be due to a transient association.

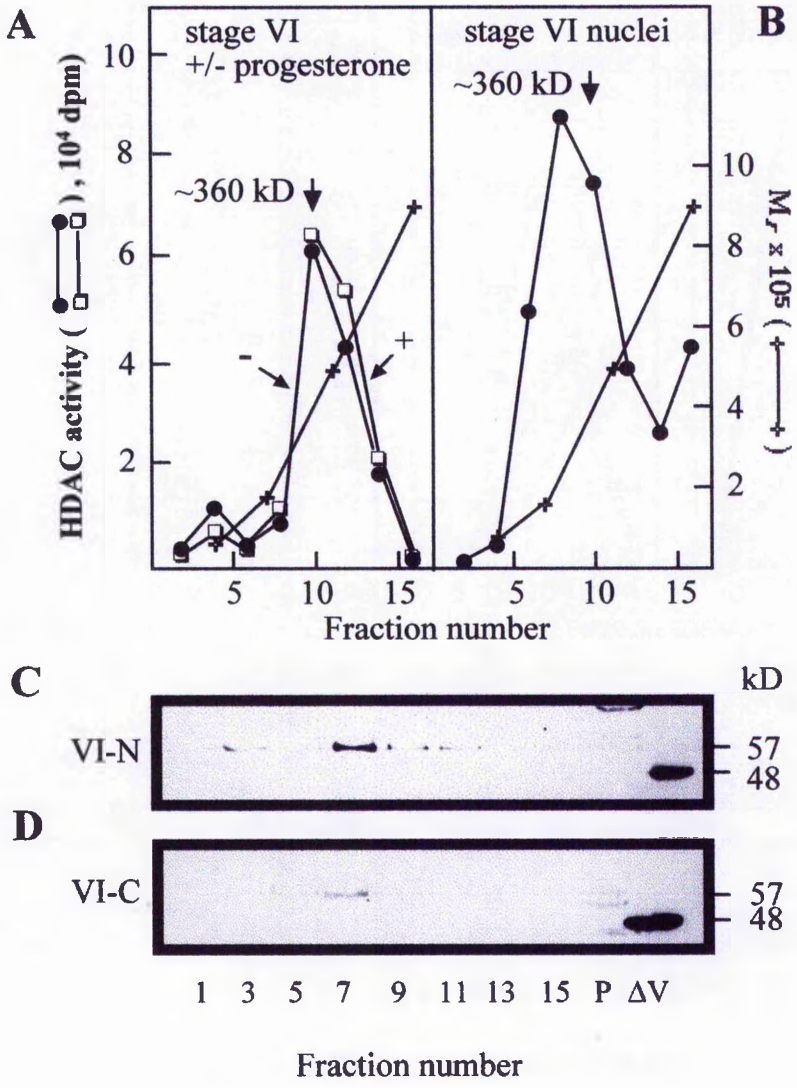



**Figure 33: Sedimentation analysis of particles containing HDACm protein and HDAC activity**: A) HDAC activity from extracts of 50 stage VI oocytes (black circles) and oocytes pretreated with progesterone (white squares) separated on glycerol gradients. Molecular weight markers shown (crosses). B) HD activity from extracts of 100 stage VI nuclei gradients (black circles). C) Immunoblot using anti-Cpep, of stage VI nuclear material from odd fractions in B). Pellet (P) and GST-HDΔV fusion protein are included in the blot. D) Same as in C) except sample stage VI cytoplasmic material from odd fractions.

## 4.4 Phosphorylation of Histone Deacetylase by CK2

*4.4.1 Potential phosphorylation sites*: HDACm has six potential phosphorylation sites that could be recognized and phosphorylated by CK2. The corresponding target site is (S/T X X D/E). Five potential sites are located within the charged tail domain of the HDACm protein (fig. 34). Also within this tail domain is a potential bipartite NLS: 438-**KKVKR**VKTEEEKEGED**KK**DV**K**-458 similar to the NLS found in the nuclear protein N1/N2 in *Xenopus* (Dingwall and Laskey, 1991). One of the five potential phosphorylation sites lies within the potential NLS (red) and could be important for regulation of nuclear import. The glutathione fusion protein called GST-HDΔV contains five of these six CK2 site and contains the entire charged tail domain (fig. 34).

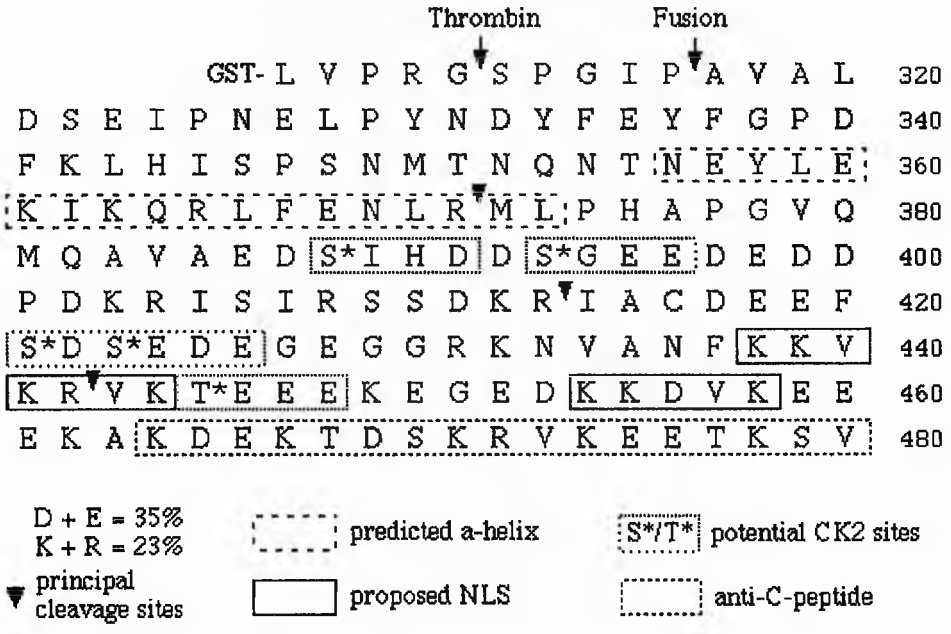



**Figure 34: Map of the glutathione fusion protein HDΔV**: The potential phosphorylations sites are marked on serines (S) or threonines (T) with the symbol *. Also marked is the potential NLS and the region recognised by anti-Cpep. A dotted box shows the position of a predicted α-helix and arrows mark the major cleavage sites predicted from proteolysis.

Phosphorylation with [$^{32}$P]γ-ATP of the GST-HDΔV fusion protein, and fusion proteins representing varying portions of HDACm, illustrates the approximate location of these CK2 phosphorylation sites. Potential sites within the fusion proteins produced from HDACm are represented in figure 35A. These represent proteins containing: the N- terminus, catalytic core and one potential CK2 site (ΔR/ΔH); the charged tail domain containing five of the potential CK2 sites (GST-HDΔV) and the majority of the HDACm excluding a small portion of the amino terminus but all six potential CK2 sites (GST-HDΔR). Incubation of these fusion proteins with nuclear extract (as a source of CK2) and [$^{32}$P]γ-ATP (see Methods, section 2.11) shows that only proteins containing the charged tail domain (GST-HDΔV-48 kDa and GST-HDΔR-85 kDa) are phosphorylated (fig. 35B). Multiple phosphorylated bands can be seen in some of the tracks due to limited proteolysis, especially with GST-HDΔV, occurring during extraction and incubation. This results in multiple bands being observed at 48, 43 and 39 kDa of GST-HDΔV due to cleavage from the carboxyl end of the fusion protein (fig. 35B). The 42 kDa protein is phosphorylated as efficiently as the 48 kDa fusion protein which means that the phosphorylation sites beyond 5 kDa from the carboxy-terminus must be phosphorylated. Labelling of some endogenous proteins can be seen in the phosphorylation gels, the histone chaperones N1/N2 at 120/115 kDa and nucleoplasmin at 35 kDa which conveniently provide internal markers of phosphorylation (fig. 35B). There were no significant differences seen in the pattern of phosphorylation when using either whole nuclear extract or isolated CK2 activity except for the presence of these endogenous substrates. Activity from both nuclei and purified CK2 were inhibited by low concentration of heparin and also showed

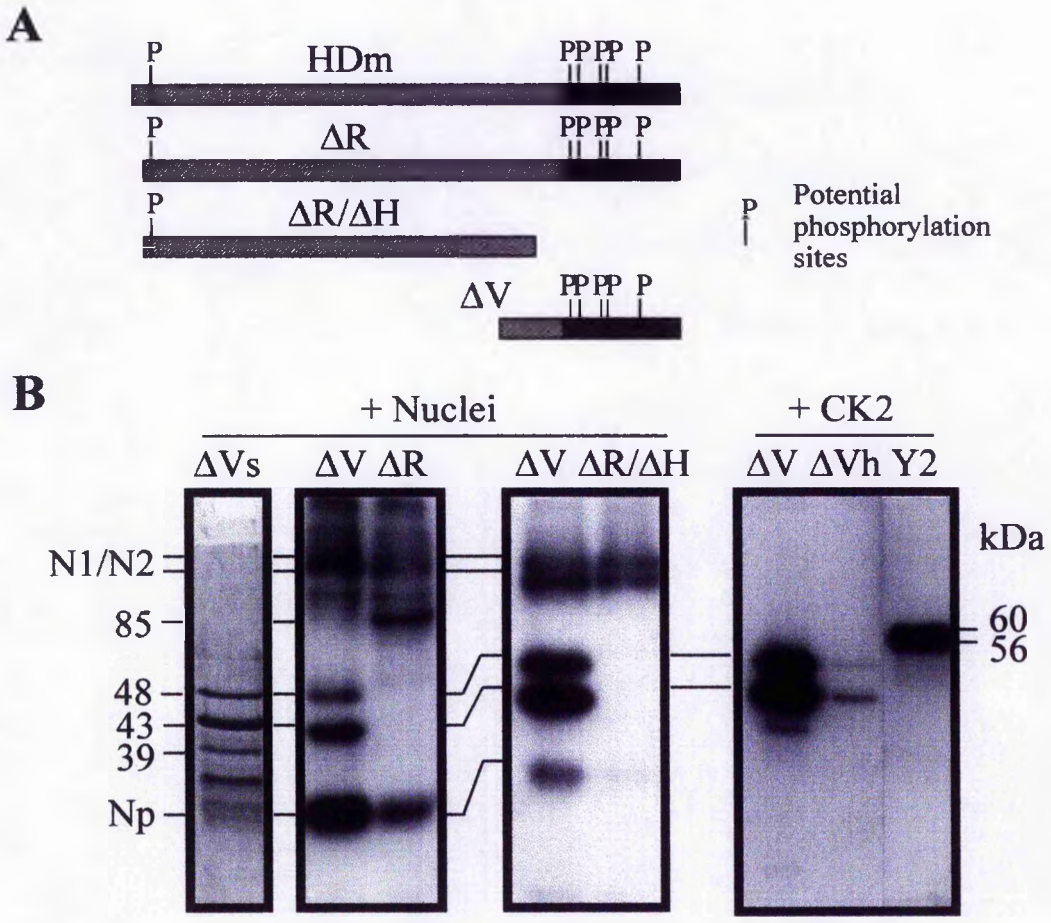



**Figure 35: Phosphorylation of HDACm fusion proteins**: A) Pictorial representation of HDACm fusion proteins GST-HDΔR, GST-HDΔR/ΔH and GST-HDΔV showing varying lengths of HDACm regions and potential CK2 phosphorylation sites. B) GST-HDΔV track shows breakdown products of the fusion protein at 43 and 39 kDa. GST-HDΔV and GST-HDΔR tracks shows the efficient phosphorylation of fusion proteins containing the carboxy-tail domain. Also represented is the breakdown product of GST-HDΔV at 43 kDa and the endogenous nuclear proteins N1/N2 and nucleoplasmin. The GST-HDΔV and GST-HDΔR/ΔH tracks shows that fusion proteins without the carboxy-tail domain do not get phosphorylated. The GST-HDΔV, GST-HDΔVh and Y2 tracks shows that GST-HDΔV phosphorylation is inhibited by heparin (h) and that the CK2 extract also efficiently phosphorylates the Y-box proteins FRGY2a/b.

phosphorylation activity towards a known CK2 substrate, FRGY2a/b (fig. 35B).

*4.4.2 Phosphorylation sites of the HDACm clone GST-HDΔV detected by peptide sequencing*: The fusion protein GST-HDΔV was phosphorylated using extracted CK2 and [$^{32}$P]γ-ATP as a label. The labeled GST-HDΔV was then digested with trypsin and sequenced using an amino acid sequencer (see Methods, section 2.12). Two peaks of radioactivity were obtained by separating the digest on fine-bore HPLC (fig. 36A). The peptides corresponding to the peak fractions were then sequenced and the material from each cycle was collected on filters and assayed for radioactivity. In peptide 1, peaks of radioactivity were observed in cycle 8 and in cycle 10 which represented the phosphorylation of two serine residues at these positions. These same results were found in two separate runs of this same material. Radioactivity was recovered from peptide 2 only during cycle 22 which meant that the serine at position 22 had been phosphorylated by CK2 (fig. 36C). The serine at position 17 was not labelled, in spite of being contained within a CK2 consensus site. The site TEEE located in the NLS was also found not to be phosphorylated under the conditions used which might discount this site as a regulator of nuclear import. However, it must be pointed out that since this site lies within the portion of the carboxyl-tail domain that tends to be lost by proteolysis, this site would be under-represented during the sequencing steps. Also, the phosphorylation of this site might be strictly controlled and these conditions might not be met by *in vitro* labeling system. It appears that only three of the potential five sites in the tail domain of HDACm are phosphorylated by CK2 under *in vitro* conditions. These sites were all serines and were located at positions 393, 421 and 423 of HDACm. The peptide separation and sequencing was carried out by Dr. Graham Kemp.

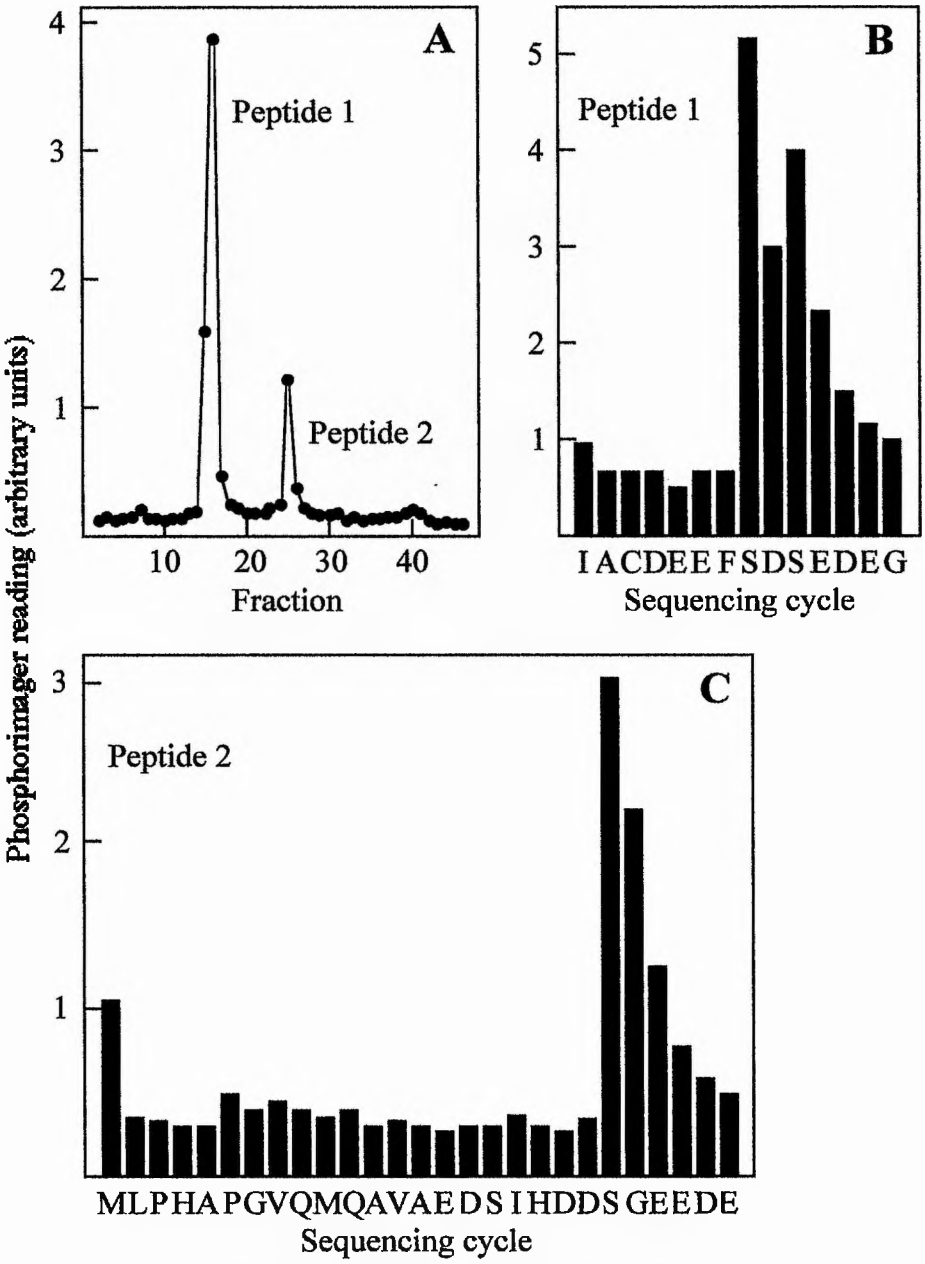



**Figure 36: Peptide sequences showing phosphorylated residues:** A) Separation of trypsin digest of phosphorylated GST-HDΔV showing peaks of radioactivity in fractions 15 and 26. B) Sequence of peptide 1 showing radioactive serine residues at positions 8 and 10. C) Sequence of peptide 2 showing radioactive serine residue at position 22.

## 4.5 Nuclear Import of HDACm

*4.5.1 Nuclear import is dependent on the presence of the C-terminal domain*: In an effort to determine if the C-terminal domain was necessary for nuclear transport of HDACm fusion proteins, labeled proteins were injected into the cytoplasm of stage VI oocytes. The complete histone deacetylase HDACm and a truncated form HDΔH were translated in reticulocyte lysate from run-off transcripts using the TnT (Promega) system incubated 2 hrs at 30°C in the presence of 10 mCi/ml of $^{35}$S-methionine (1,000 Ci/mmol, Amersham) by Dr. John Sommerville. Transcription was from either the complete cDNA template (HDACm) in pBluescript (Promega) or from a 3'truncation at the Hind III site (HDΔH), to produce a protein missing the charged tail domain. The labelled proteins were separated from unincorporated label using Sephadex-G50 spin columns. Approximately 20 µl (~50pg) of labelled protein was injected into the cytoplasm of stage VI oocytes which were left to incubate at room temperature (see Methods, section 2.13). At specified time points, oocytes were collected and the nuclei were removed under oil (see Methods, section 2.9). Five nuclei and the corresponding five cytoplasms for each time point were collected on 3MM filters. Protein on the filters was precipitated by placing the filters in 10% trichloroacetic acid (TCA), the filters were washed thoroughly in 5%TCA and the radioactivity was counted with a scintillation counter.

The injection of the $^{35}$S-labelled HDACm proteins into the cytoplasm of *Xenopus laevis* oocytes resulted in a 10-fold concentration of the protein in the nucleus after a period of 24 hrs (fig. 37). On injecting the truncated form of the protein (HDΔH), which lacks 16 kDa off the terminal end of the charged tail domain, it failed to concentrate substantially in the nucleus compared to the HDACm protein

(fig. 37). This indicates that at least part of the carboxyl-tail domain is needed for efficient transport into the nucleus. The fact that the putative NLS is located in this region lends support to the notion that it is used for nuclear uptake of HDACm.

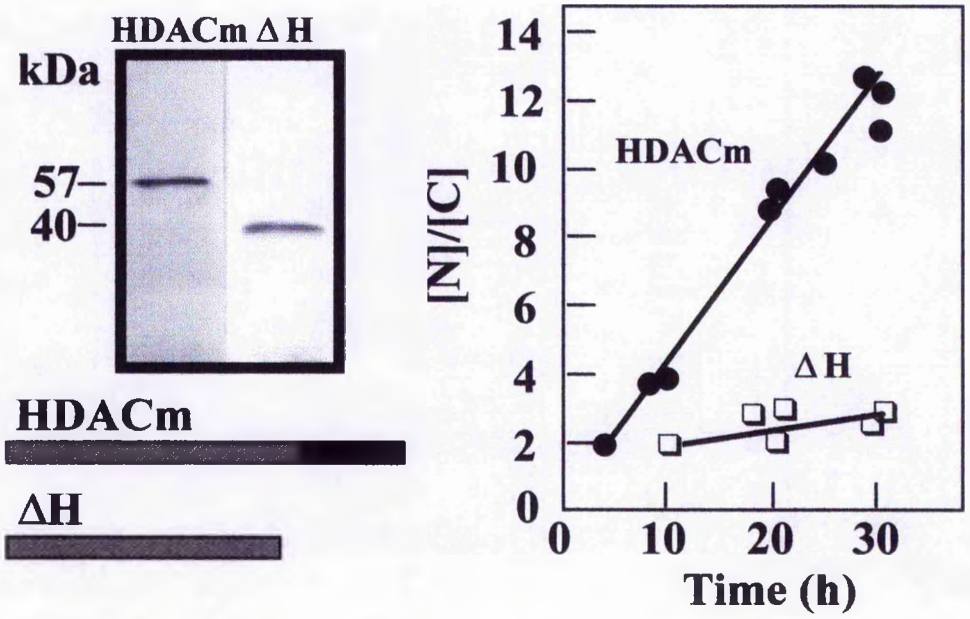



**Figure 37: Nuclear import of $^{35}$S labelled HDACm and ΔH**: The picture on the left is an autoradiograph depicting the $^{35}$S labelled forms of HDACm (57 kDa) and GST-HDΔH (40 kDa), below is a pictorial representation of each, the black block representing the C-terminal domain. The graph on the right plots the ratio of nuclear concentration to cytoplasmic concentration of injected $^{35}$S labelled HDACm (black circles) over a 30 hour period. This same ratio of nuclear to cytoplasmic concentration of injected $^{35}$S labelled protein, (ΔH) missing the C-terminal domain, is represented by the white squares.

*4.5.2 Nuclear import is dependent on the presence of the putative NLS*: Two forms of HDACm fusion protein GST-HDΔV and GST-HDΔV-5, which represents the GST-HDΔV with 5 kDa lost from the C-terminal domain, were phosphorylated with CK2 (using [$^{32}$P]γ-ATP) and injected (~50 pg) into stage VI oocytes (see Methods, section 2.11 and 2.13). These two forms were injected to determine whether the last 5 kDa, which contains the putative bipartite NLS, was actually necessary for nuclear uptake of the fusion protein. The fusion protein was labelled with [$^{32}$P]γ-ATP primarily to enable the protein to be localised, but another reason was to see if the phosphorylated protein had improved import kinetics compared to the $^{35}$S labelled injection experiments. When injections were completed, oocytes were incubated at room temperature, after which, at specific time points, sets of five oocyte nuclei and cytoplasms were separated (see Methods, section 2.9). These nuclei and cytoplasms were solubilized and loaded onto SDS-PAGE gels after which gels were dried down for autoradiography (see Methods, section 2.6). The protein gel was also placed into the InstantImager (Packard) to enable a accurate, specific quantification of labelled protein of each sample (see Methods, section 2.15).

The injection of these proteins showed that only the phospolabelled protein containing the complete C-tail domain (GST-HDΔV -48 kDa) accumulated in the nucleus (fig. 38A). The truncated form, GST-HDΔV-5 (43 kDa), is not detected in the nucleus and after 50 hrs phospholabel is completely lost from the cytoplasm (fig. 38A and B). This loss could be due either to degradation of the protein or to loss of the label by dephosphorylation. The kinetics of nuclear uptake of the GST-HDΔV fusion protein showed a rapid and efficient translocation: over 80% of the protein is isolated within the nucleus 10 hrs after injection (fig. 38B). Total protein uptake in

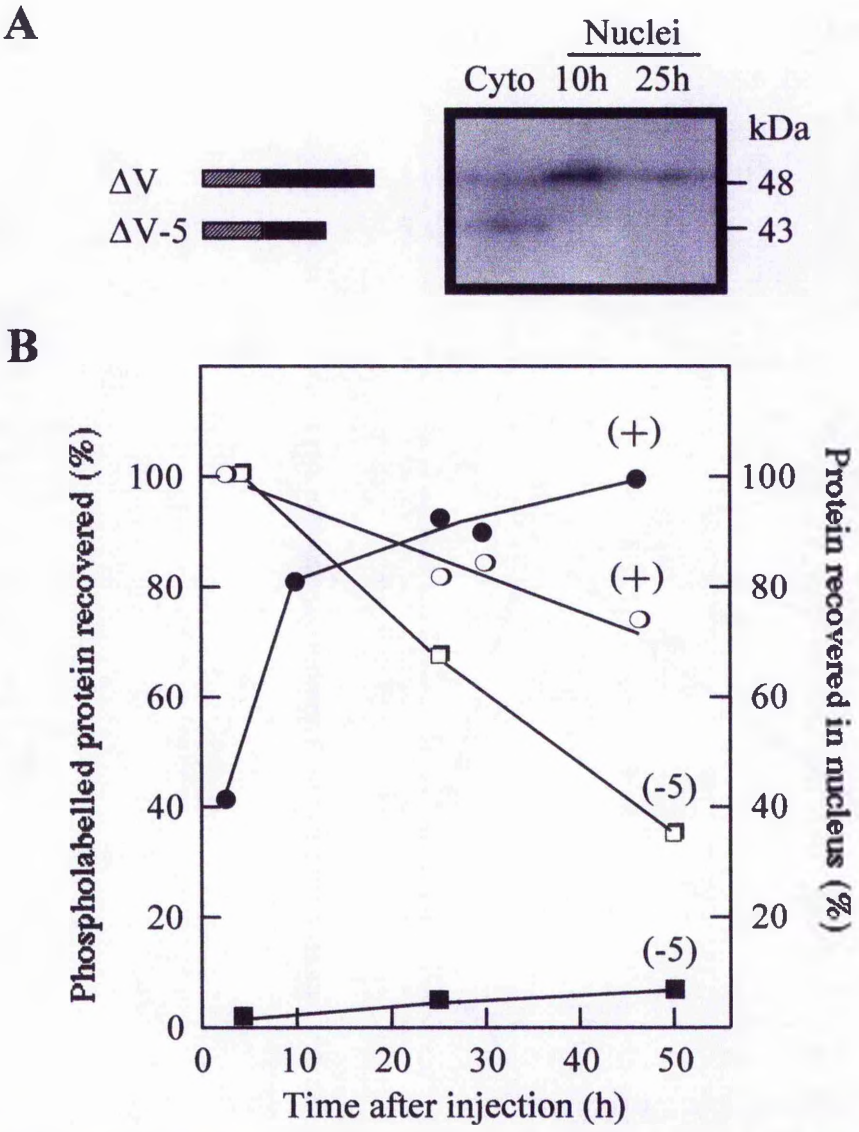



**Figure 38: Nuclear import of GST-HDΔV and GST-HDΔV-5**: A) Autoradiograph showing a GST-HDΔV band at 48kDa in the nucleus (from 10 oocytes) at 10 and 25 hrs after injection. The cytoplasmic sample (from 2 oocytes) shows both the GST-HDΔV and GST-HDΔV-5 bands at 2 hrs. B) Graph depicting the efficiency of transport of the GST-HDΔV (black circles) after 10 hrs over 80% of the protein is recovered in the nucleus. The ΔV-5 protein (black squares) is not efficiently transported to the nucleus. The amount of degradation is represent by the white circles for GST-HDΔV and the white squares for GST-HDΔV-5.

these experiments amounts to more than 1 ng/oocyte, which compares favourably with a synthetic rate of core histones in full-grown oocytes of 0.5ng/h.

*4.5.3 Nuclear uptake is improved by phosphorylation with CK2*: The GST-HDΔV fusion protein was injected into the cytoplasms of stage VI oocytes, after treatment with inhibitors of CK2, to determine if the kinetics of nuclear translocation were affected by preventing phosphorylation. Before injection, some oocytes were bathed in the kinase inhibitors DRB and quercetin to ensure that no phosphorylation of the GST-HDΔV fusion protein would occur in the cytoplasm. Oocytes were also treated with the inhibitors of CK2, 0.16μM of quercetin (see figure 23 for sensitivity), and 50 μM DRB (see figure 22 for sensitivity), for ~ 2hrs. prior to injection. Rutin, an inactive analogue of quercetin served as a control. The GST-HDΔV fusion protein (~50 pmols) was injected into sets of 100 stage VI oocytes and left at room temperature for ~20 hrs (see Methods, section 2.13). The GST-HDΔV fusion protein had been treated with alkaline phosphatase for 2 hrs prior to use in injection experiments to ensure that no sites were pre-phosphorylated. Alkaline phosphatase was removed by extensive washing of the GST-HDΔV bound to glutathione-Sepharose beads (see, Methods, section 2.5). After incubation of the injected oocytes for 20 hrs, the nuclei (20 for each sample) were isolated from the cytoplasms and proteins were separated by SDS-PAGE gel (see Methods, section 2.9 and 2.6). Separated proteins were transferred to nitrocellulose and immunoblotted with the anti-HDACm antibody (see Methods, section 2.7).

The injected oocytes that were not treated with inhibitors of CK2 or were treated with rutin showed rapid and efficient nuclear import of the 48 kDa fusion protein GST-HDΔV (fig. 39A). The oocytes treated with the inhibitors quercetin and

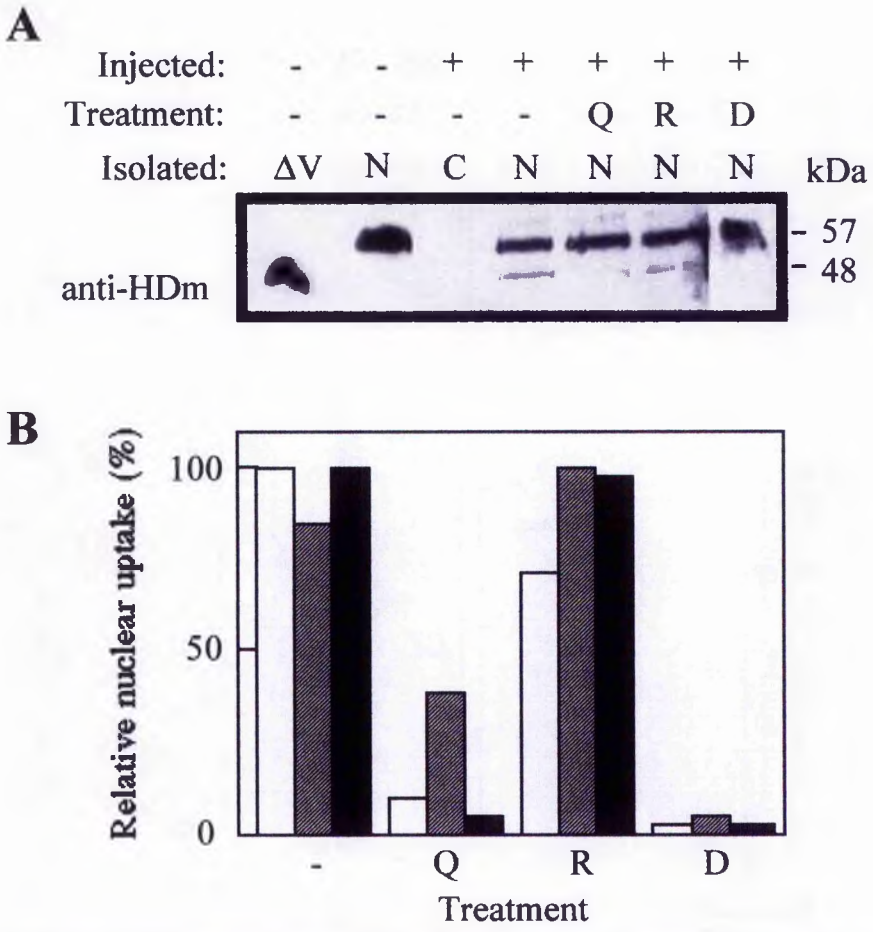



**Figure 39: Effects of the injection of GST-HDΔV into the cytoplasms of Stage VI oocytes treated with kinase inhibitors**: A) An immunoblot of isolated nuclei injected with GST-HDΔV fusion protein. The 57 kDa band is endogenous HDACm and the 48 kDa band is injected GST-HDΔV that has been transported to the nucleus. Injected = whether they were injected with GST-HDΔV (+ or -), Treatment = if they were treated with inhibitors: Q= quercetin, R= Rutin, and D= DRB, and Isolated = what fraction was examined: N= nuclei and C= cytoplasm. B) Graph representing the relative % of the injected GST-HDΔV found in the nucleus. The symbol (-) means not treated, (Q) means treated with quercetin, (R) means treat with rutin, and (D) means treated with DRB. Each set of bars (white, black and striped) represents a different experiment.

DRB showed drastically reduced import of the GST-HDΔV fusion protein compared to the untreated and rutin control (fig. 39A). The 57 kDa band, shown in figure 39A, represents endogenous histone deacetylase that is also stained with the anti-HDACm antibody. The control nuclear extract, in this same figure, shows that no immunoreactive bands are present at 48 kDa, if the oocytes are not injected. The C track (fig. 39A) represents isolated cytoplasm, which shows that no detectable GST-HDΔV remains in the cytoplasm after 20 hrs. The graph (fig. 39B) shows the results of scanning immunoblots from these separate experiments. In all instances the highest levels of nuclear GST-HDΔV were found in untreated and rutin-treated samples. Quercetin gives an average of only 15% relative nuclear uptake, and DRB gave less than 5% nuclear uptake.

## 4.6 Binding of α-Importin to GST-HDΔV is Influenced by Phosphorylation

*4.6.1 α-importin binds most efficiently to unphosphorylated GST-HDΔV*:

One further question that had to be addressed was how the phosphorylation of the GST-HDΔV fusion protein affected its nuclear import. One idea was to study how this phosphorylated form of HDACm associated with a well known nuclear transport protein, α-importin. If it could be determined that phosphorylated forms of GST-HDΔV bound more readily than the unphosphorylated form, then a particular mechanism could be proposed. But as it happens, this was not what was observed, but actually quite the opposite was seen.

The fusion protein GST-HDΔV was bound to glutathione-Sepharose beads, but instead of eluting the protein with excess glutathione it was left bound to the beads (see Methods, section 2.5). One sample of purified GST-HDΔV was

phosphorylated while still bound to the glutathione-Sepharose beads. This was done by adding the equivalent of 5 stage VI nuclei, and 1 mM ATP and leaving to incubate at room temperature for 1 hr (see Methods, section 2.11.4). An SN100 was made from homogenised stage V/VI oocytes (see Methods, section 2.9). This fraction contained α-importin as detected by immunoblotting and was used as the source for the binding assays. The SN100 was added to the GST-HDΔV bound to the glutathione-Sepharose beads and incubated at room temperature for 1 hour with mild agitation. After the 1 hour unbound protein was removed and the beads were washed 5 times in cold TBS to remove any unbound protein. The GST-HDΔV and bound α-importin were removed by eluting the sample with 10mM reduced glutathione (see Methods, section 2.5). The eluted proteins were separated by SDS-PAGE, then transferred to nitrocellulose and immunoblotted with anti-α-importin (provided by Dr. Dan Gorlich), (see Methods, section 2.6 and 2.7).

This procedure was carried out with three forms of the GST-HDΔV fusion protein. The first was the complete unphosphorylated GST-HDΔV, the second was the complete GST-HDΔV fusion protein phosphorylated with CK2 from stage VI nuclei and the third was the unphosphorylated GST-HDΔV-5 fusion protein (lacking the C-terminal 5kDa portion). Figure 40 shows that the unphosphorylated GST-HDΔV fusion protein gave the most efficient binding of α-importin (60 kDa) while the phosphorylated form of GST-HDΔV gave relatively poor binding of α-importin. The GST-HDΔV-5 clone also showed poor binding of α-importin, but this might be expected since this protein is missing the NLS, the region to which α-importin most likely binds. No binding of α-importin is seen with glutathione alone, and no

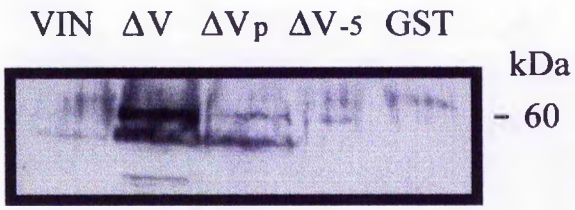



**Figure 40: Effect of phosphorylation on α-importin binding to GST-HDΔV**: The first track is an immunoblot of stage VI nuclei (VIN) in order to represent the amount of α-importin added from the source of CK2. The second is an immunoblot of the binding of α-importin to unphosphorylated GST-HDΔV. The third track is an immunoblot of the binding of α-importin to phosphorylated GST-HDΔV(ΔVp). The fourth is an immunoblot of the binding of α-importin to GST-HDΔV missing 5 kDa from the carboxyl-tail domain (ΔV-5). The fifth tract is an immunoblot of the binding of α-importin to glutathione S transferase.

significant amounts of native α-importin blotted from the amounts of stage VI nuclei used for phosphorylation.

*4.6.2 Phosphorylation promotes the release of α-importin binding to GST-HDΔV*: Since there seemed to be no enhanced binding of α-importin to GST-HDΔV after phosphorylation of the CK2 sites in the GST-HDΔV, an alternative was considered; that phosphorylation enhanced release of the bound α-importin. This was tested by binding α-importin to the bound GST-HDΔV as before, but then phosphorylating the GST-HDΔV and α-importin with CK2 afterwards, to see if the addition of phosphates would disrupt the interaction of these two proteins.

The GST-HDΔV was bound to glutathione-Sepharose beads as before, and then incubated with SN100 using the same procedures as stated in the previous section. Components from the SN100 were left to bind and then the beads were washed several times with cold TBS. The beads were raised in protein kinase buffer; then stage VI nuclei and 1 mM ATP were added and the mix was left to incubate at room temperature for 1 hour. Samples were also incubated with inhibitors of CK2 at the same time to see if the phosphorylation-induced release could be inhibited. The

inhibitors were added at final concentrations of: 50μM DRB, 0.16μM of quercetin, and 0.16μM of rutin as a control. After this incubation with gentle agitation, the unbound supernatant was removed from the beads and was acetone-precipitated and loaded onto a protein gel (see Methods, section 2.6). The separated proteins were transferred to nitrocellulose and blotted with anti-α-importin (see Methods, section 2.7).

Control samples were added to this gel as well, one being stage VI nuclei alone (VI in fig. 41) and also GST-HDΔV bound to beads, but with no protein kinase or inhibitors added. ATP was added and incubated as with the other samples (B in fig. 41). The second track does show some release of α-importin with 1 mM ATP added which might mean that ATP may aid disrupting activity by components already bound to the beads, but the amount released is less than that released after adding CK2. The sample with ATP and nuclear extract (third track in fig. 41) shows a large release of α-importin after phosphorylation, which would seem to suggest that phosphorylation with CK2 may promote efficient release of the NLS of GST-HDΔV from α-importin. The samples that were also incubated with CK2 inhibitors gave mixed results. The DRB reaction showed complete blockage of release of α-importin, but the quercetin reaction (Q in fig. 41) showed a relatively small change in the amount released compared to that of rutin (R in fig. 41), the inactive analogue of quercetin.

The DRB inhibition of release was repeated and this time examining the remaining α-importin still bound to the beads. The procedure was the same as in the previous experiment except after the bound α-importin was eluted, the beads were

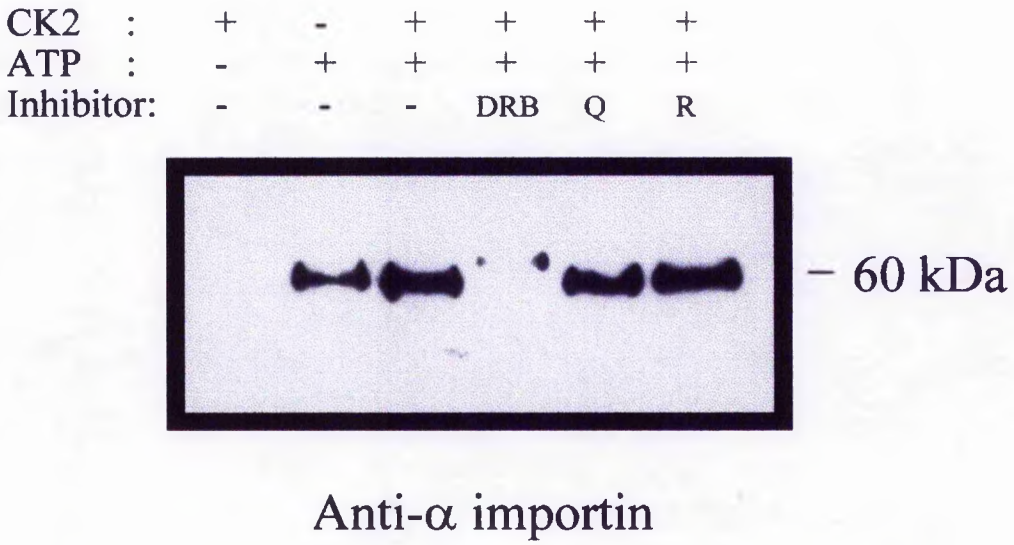



**Figure 41: The effect of phosphorylation on the release of α-importin from GST-HDΔV**: The first track is a immunoblot of proteins from 10 stage VI nuclei with anti-α-importin. The second track is the release of α-importin from GST-HDΔV after incubation with 1 mM ATP. The third track is the release of α-importin after incubation with stage VI nuclei and 1 mM ATP. The fourth track is the release of α-importin after incubation with stage VI nuclei, 1 mM ATP and 50μM DRB. The fifth track is the release of α-importin after incubation with stage VI nuclei, 1 mM ATP and 0.16μM of quercetin. And the sixth track is the release of α-importin after incubation with stage VI nuclei, 1 mM ATP and 0.16μM of rutin.

stripped using 1% SDS and 0.1mM DTT. This was done to ensure that the α-importin that was not being released from the GST-HDΔV by the incubation conditions was still bound to the GST-HDΔV on the beads. Also instead of using ATP, GTP was used at the same concentration of 1 mM. The use of GTP was to check that the protein kinase could use either GTP or ATP to promote release. These samples were acetone precipitated and the proteins were separated by SDS-PAGE, after which they were transferred to nitrocellulose paper and immunoblotted with anti-α-importin.

Again, figure 42 shows that 50 μM DRB inhibits the release of α-importin compared to the track containing CK2 and GTP without the inhibitor. However, the effect of DRB inhibition is much less than seen in the previous experiment. One

possibility is that this could be due to the use of GTP instead of ATP as the phosphate donor. The relative amount of α-importin retained on the beads is quite high in the DRB track compared to that of the uninhibited track, confirming that release had indeed been inhibited. The tentative conclusions from these experiments is that inhibition of CK2 activity results in the lack of phosphorylation of the HDACm tail domain and hence failure to disrupt the binding of α-importin.

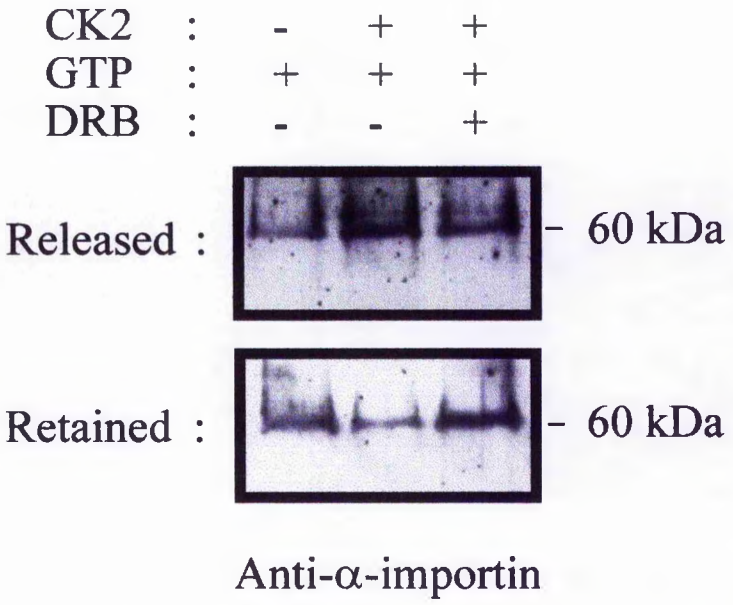



**Figure 42: Release and retention of α-importin after phosphorylation**: The samples are identical for top and for bottom blots, except the top is the released fraction and bottom is the retained fraction. The first track is a control showing release and retention after the addition of 1 mM GTP and 1 hour incubation. The second track shows release and retention after the addition of CK2 enzyme, 1 mM GTP and 1 hour incubation. The third track shows release and retention after the addition of stage VI nuclei, 1 mM GTP, 50mM DRB and 1 hour incubation.

An apparent difference seemed to exist in the α-importin release experiments, between using GTP and using ATP as a co-substrate. To determine if there was any basis to this observation, a titration was performed using ATP and GTP in the absence of added CK2. This was to discover if ATP and GTP had any direct effect on binding, and if increasing concentrations would effect the level of α-importin release.

The procedures were similar to the previous experiments. GST-HDΔV was bound to glutathione-Sepharose beads and then washed with cold TBS. The GST-HDΔV beads were then incubated with SN100 and washed again with TBS. The samples were then incubated with increasing concentrations of ATP and GTP. The concentrations for both ATP and GTP were 0, 0.05, 0.1, 0.5 and 1 mM. After incubation with ATP or GTP the released material was collected, treated as in the previous experiment, and immunoblotted with anti-α-importin.

The results of the immunoblots show that increasing concentration of ATP promotes an increased level of α-importin release (fig. 43). Increasing levels of GTP did not raise the release of α-importin above the basal level. The differences between these two substrates is puzzling, since there should be no difference in the ability of CK2 to use either of these phosphate donors. The difference may lie in the ability of α-importin to use GTP and not ATP for the catalysis of its transport function. But this does not explain why ATP is more efficient at promoting release of α-importin than GTP. The observation that ATP and GTP can promote a basalm level of release without the addition of a source of CK2 activity also requires explanation. The protein α-importin might be sensitive to the presence of ATP or

GTP as part of the disassociation reaction, but more likely, it could be that the reaction was contaminated with CK2, which was bound to the GST-HDΔV from the SN100 extracts. Another possibility is that slow release is due to GST-HDΔV breakdown, thus release of some bound α-importin may occur as the tail domain is lost.

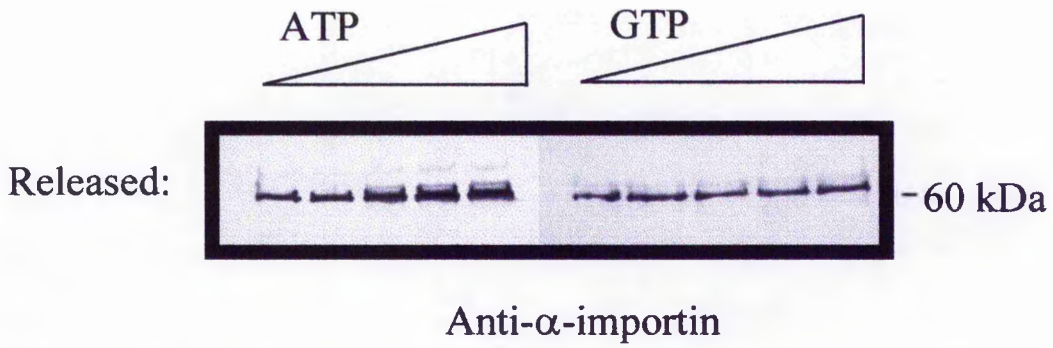



**Figure 43: The effect of increasing concentrations of ATP and GTP on the release of α-importin from GST-HDΔV**: The left blot is an immunoblot with anti-α-importin of release after incubation with increasing concentrations of ATP. The right blot is an immunoblot with anti-α-importin of release after incubation with increasing concentrations of GTP. The range of concentration is 0, 0.05, 0.1, 0.5 and 1mM for both ATP and GTP.

### 4.7 Summary

This chapter illustrated the findings that the HDACm protein accumulates mainly in the nucleus during oocyte maturation to reach a relative peak at stage VI of oogenesis. The rate of accumulation and location of protein is similar to that of CK2, which also reaches its peak in stage VI nuclei. The histone deacetylase activity was measured, and corresponded on rate sedimentation analysis to a protein complex of 360 kDa which is larger than the complex found for CK2. Histone deacetylase activity seems to be sensitive to treatment with alkaline phosphatase. Thus it seems

that the phosphorylation state could be a potent regulator of the deacetylase activity of the complex.

Nuclear import of HDACm fusion proteins was shown to be dependent on the presence carboxyl-tail domain which contains the majority of the CK2 sites present in HDACm. The nuclear import rate of HDACm fusion proteins was also shown to be increased, during injection into oocytes, by the phosphorylation of these sites by CK2. This illustrates another potential regulatory function of CK2 on the enzyme histone deacetylase. How the phosphorylation state of histone deacetylase affects nuclear import was examined by studying the binding of a well-know nuclear transport protein, α-importin, with the potential nuclear localizaton signal of HDACm. Phosphorylation by CK2 seemed to disrupt the binding of α-importin to the region that contained the NLS. The interpretation favoured here is that phosphorylation might lead to improved protein disassociation once the complex has reached the inside of the nucleus. Release of bound proteins was also affected by increased levels of ATP. Why this occurs is not clear, but high ATP concentrations, approximating to that found in nuclei *in vivo*, might be one of the prerequisites for release of transported protein by α-importin.

# Chapter 5: Results

# mRNP-Associated Protein Kinase Activity

## 5.0 Aim

The mRNP particle has been studied for many years because of its importance in the long term storage of maternal mRNA. The storage of mRNA is necessary to continue the production of important proteins after fertilization, when continuous cell divisions mean that there is no time to transcribe genes. Several of the protein constituents of mRNP have been identified and three of the major components are FRGY2a and b (Y-box proteins), and RNA helicase p53. In addition an associated protein kinase activity, which is thought to be CK2, has been described (Cummings and Sommervile, 1988). This chapter will study the phosphorylation of the proteins FRGY2a/b in an attempt to define what type of kinase they are phosphorylated by, and what possible effect this phosphorylation could have on their function.

## 5.1 mRNA-Associated Proteins FRGY2a and b

The proteins FRGY2a and FRGY2b are major RNA binding proteins of mRNP particles. Their binding to RNA has been widely studied, and the regulation of their binding is thought to influence the translation of stored mRNP in immature *Xenopus* oocytes. The two proteins have a very similar amino-acid sequence (fig. 44) containing virtually identical cold shock domains (CSD) thought to be instrumental in their ability to bind to RNA. They also contain many potential CK2 phosphorylations sites which might suggest a regulatory pathway governed by this enzyme. It has been considered, in the past, that the phosphorylation state of these two proteins might

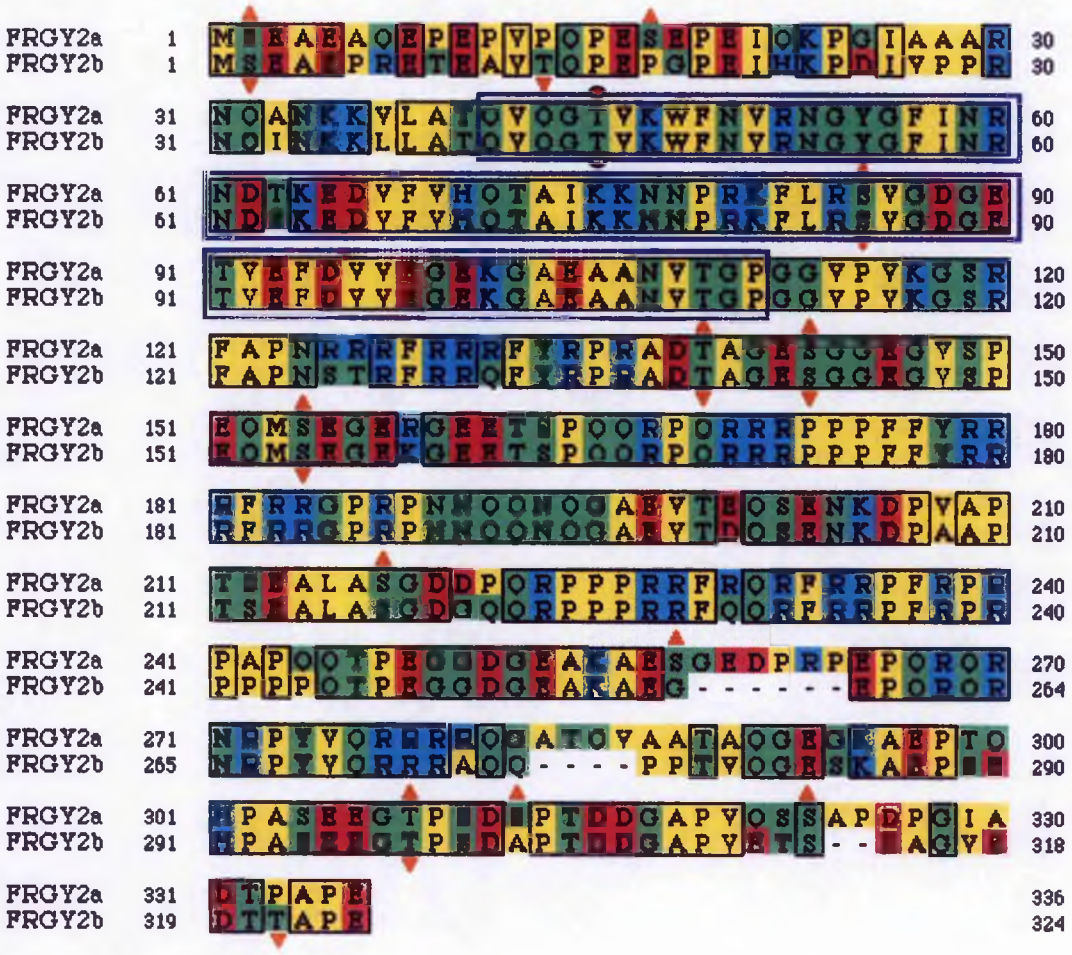



**Figure 44: FRGY2a and FRGY2b sequence alignment**: Areas of sequence homology are boxed in black. The CSD domain is boxed in blue. Red shading represent acidic residues, blue represents basic residues, yellow represents non-polar residues and green represents uncharged polar residues. The orange triangles represent potential CK2 phosphorylation sites. The red circles represent PKC phosphorylation sites.

affect their binding to RNA, but this has yet to be fully explored. There is, associated with these mRNP particles, a protein kinase thought to be protein kinase CK2, which would play a regulatory role (Sommerville, 1992).

**5.2 Phosphorylation of FRGY2a/b by a Nuclear Protein Kinase**

Purified FRGY2a/b was used as a substrate for stage VI nuclear extract in an attempt to show that the phosphorylation of Y-box proteins resembled that of other CK2 substrates. These two Y-box proteins were purified from immature oocytes using a method of successive heat treatments in order to precipitate contaminating proteins, while leaving the Y-box proteins soluble (see Methods, section 2.18.2). After purification, these Y-box proteins were phosphorylated using stage VI nuclei and [$^{32}$P]γ-ATP under varying conditions which might influence CK2 activity. The phosphorylation assays were 10μl reactions which consisted of: 1μl of stage VI nuclear kinase (equivalent to 1 nucleus), 1μl of [$^{32}$P]γ-ATP (0.033mM final), 1μl of 10 x kinase buffer (final: 20mM Tris-HCl, (pH.7.4), 10mM $MgCl_2$, 1mM DTT, and 100mM KCl), 2μl of the substrate Y-box protein (~0.5μg), 1μl of various phosphorylation conditions (diluted inhibitors or salt concentrations) and 4μl of $dH_20$. These reactions were left to incubated at room temperature for ~1 hr, after which 10μl of SDS-PAGE buffer was added to each reaction and the samples loaded onto an SDS-PAGE protein gel. Gels were stained with Coomassie blue and destained, dried onto 3M paper and setup for autoradiography (see Methods, section 2.6).

The results of these various phosphorylation assays are shown in figure 45. The first gel shows the effect of varying concentrations of NaCl, KCl and $Mg^{2+}$ ions on the phosphorylation of the Y-box proteins by nuclear protein kinase (fig. 45A). As seen in previous assays for the GST-HDΔV substrate (fig. 25, chapter 3), the phosphorylation of the Y-box proteins (of 60 and 56 kDa) by nuclear protein is sensitive to increasing salt and $Mg^{2+}$ concentrations. However,

the phosphorylation of the Y-box proteins is not completely inhibited even in high concentrations of NaCl and KCl (fig. 45A: 0.4mM NaCl and KCl). The Y-box proteins are still phosphorylated even when all phosphorylation of endogenous proteins is eliminated. This could be explained by a high binding affinity that the protein kinase may have for this particular substrate, which is stable even in high salt concentrations. The phosphorylation is somewhat reduced after the addition of 2mM EDTA which reduces the amount of divalent cations such as $Mg^{2+}$, a required cofactor of CK2. The phosphorylation is improved several-fold after the addition of $MgCl_2$ to a final concentration of 20mM (last track in fig. 45A).

The next assay was to determine if the kinase that was phosphorylating the Y-box proteins was sensitive to the CK2 inhibitors. The inhibitors used were DRB, quercetin, rutin (as a negative control) and heparin, (all in increasing concentrations), (fig. 45B). The first track (-N) is a negative control to show that the Y-box proteins themselves have no intrinsic protein kinase activity. The second track (-) is a control representing the phosphorylation of the substrate proteins by added nuclear extract. The following tracks all represent addition of inhibitors to the phosphorylation reaction. The track (Q) represent the phosphorylation with 20 ng/ml of quercetin added; this shows total inhibition of phosphorylation. The next track (R) is the addition of rutin, the inactive analogue of quercetin, used here as a negative control. The addition of 20ng/ml of rutin fails to inhibit phosphorylation of the Y-box proteins by the nuclear extract. The addition of DRB, seen in the (D) track, also shows efficient inhibition of phosphorylation, demonstrating the sensitivity of Y-box protein phosphorylation to this CK2 inhibitor. The next three tracks represent a serial

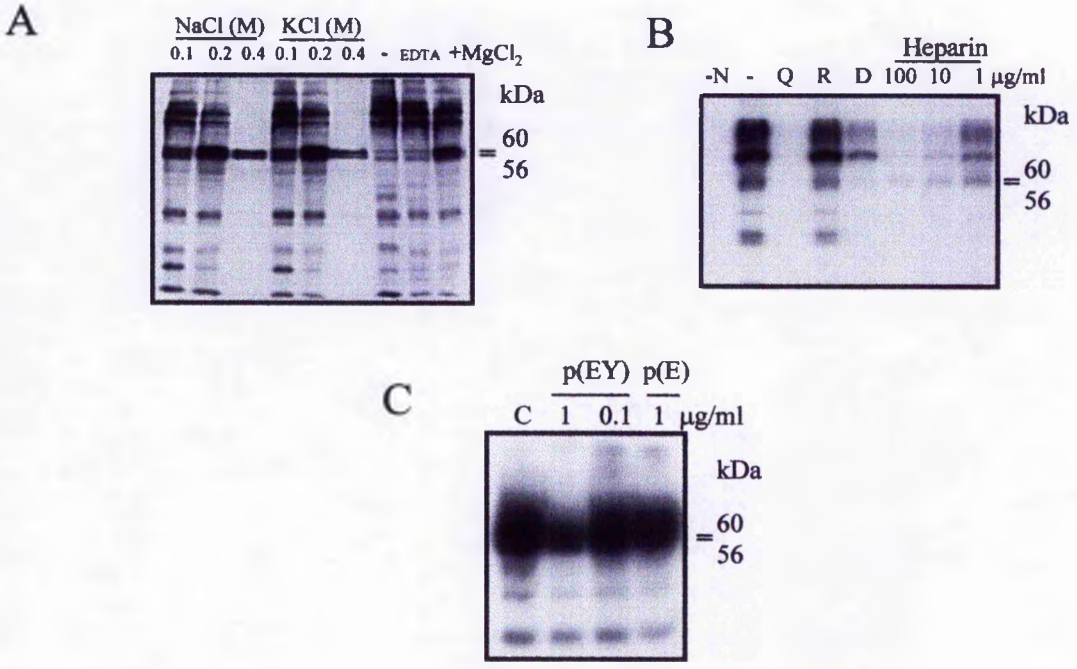



**Figure 45: Phosphorylation of Y-box proteins by protein kinase from stage VI nuclei**: A) Phosphorylation of Y-box protein under varying salt concentrations. The first three tracks: NaCl from 100mM-400mM. The next three tracks: KCl (100mM-400mM). The final three tracks: no addition of $MgCl_2$, addition of 2mM EDTA, and addition of an extra 10mM $MgCl_2$ to the reaction mixture. B) Phosphorylation inhibition of Y-box proteins using various CK2 inhibitors. The first track labeled (-N) is a negative control, representing Y-box proteins with no protein kinase added. The next track (-) is a positive control showing normal phosphorylation of Y-box proteins by nuclear kinase. The 3rd and 4th tracks show the effect of 20ng/ml of quercetin and rutin (Q and R). The 5th track shows the effect of the addition of 50µM/ml of DRB. The 6th-8th tracks show a serial dilution of heparin (100µg-1µg/ml). C) Phosphorylation inhibition of Y-box proteins by the addition of a competitive inhibitor poly(EY). The first track is a control phosphorylation of Y-box proteins (60 and 56 kDa). The second track is with 1µg/ml of poly(EY) added. The third track is with 0.1µg/ml of poly(EY) added. The fourth track is with 1µg/ml of poly(E) added.

dilution of the inhibitor heparin. The additions are 100, 10, and 1 μg/ml of heparin in respective tracks. The sensitivity of phosphorylation is evident in the higher concentrations of heparin added (100 and 10 μg/ml), but some inhibition of phosphorylation is also observed with the lowest concentration of 1μg/ml. This result also suggest that the kinase activity phosphorylating the Y-box protein is in fact protein kinase CK2. The final assay is a reaction involving the phosphorylation of Y-box proteins with the nuclear kinase in the presence of a synthetic peptide containing multiple tyrosine sites and glutamic acid sites, poly(EY), to act as a competitive inhibitor. This substrate binds very efficiently to CK2 and thus should reduce the phosphorylation of the Y-box proteins, if they are indeed being phosphorylated by CK2. A negative control was also used, namely the addition of poly-glutamic acid,
poly(E). The results show (fig. 45C) that the poly(EY) does partially inhibit phosphorylation of the Y-box proteins. The poly(E), on the other hand, has no effect on phosphorylation. This suggests that the kinase involved in this phosphorylation recognises acidic residues surrounding tyrosine sites, which is a characteristic of CK2.

**5.3 Endogenous mRNP Protein Kinase Activity**

*5.3.1 Purification of protein kinase activity from mRNP*: The purification of mRNP kinase was a multi-step process involving separation of material on two types of columns. The flow chart in figure 46 shows the purification steps taken to isolate the mRNP kinase activity. The absorbance during each of the column steps was measured at a wavelength of 254nm by a flow spectrophotometer. Although this records more efficiently the RNA present, peaks of eluted protein

are also detected. mRNP particles were isolated from a homogenate of previtellogenic ovary (~0.5g), passed through an oligo (dT) cellulose column (see Methods, section 2.18.1). The column binding buffer used was 20mM Tris-HCl (pH.8), 2mM $MgCl_2$ 1mM DTT and 200mM NaCl. The homogenised ovary (2.5 ml) was Freon extracted and spun at 10,000rpm for 15 min to separate the yolk, pigment and lipids from the soluble proteins. The supernatant (~2 ml) from this spin was then spun at 100g for 3 hrs to separate the

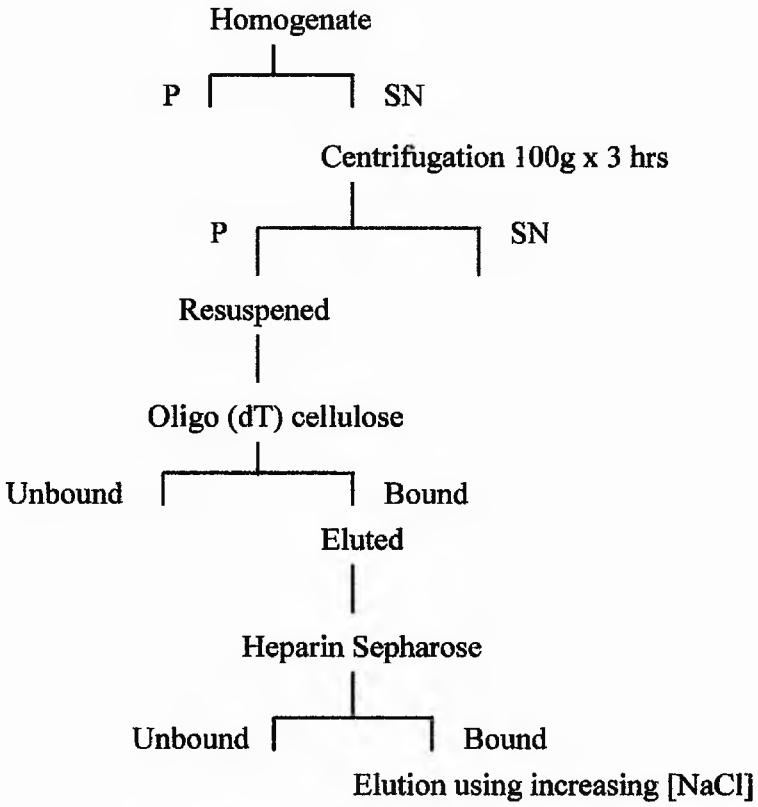



**Figure 46: Purification of mRNP-associated protein kinase**: A flowchart of the purification steps needed to obtain kinase activity from mRNP particles. Starting with homogenate of previtellogenic oocytes, which was taken through a series of column chromatography steps. The columns used were oligo (dT) Cellulose and heparin-Sepharose.

ribosomes mRNP particles from smaller protein-containing complexes. This pellet containing the mRNP particles was solubilized in ~1ml column binding

buffer and then passed over the oligo (dT) cellulose column. The solubilized pellet fraction was passed over the column three times after which the unbound material (A)- was removed and the column was washed with the column binding buffer until the absorbance trace returned to the baseline (see fig. 47). The column was then treated with warm $dH_20$ (~3 ml), to release the poly (A)+ fraction, until the absorbance trace returned again to the base line (fig. 47). The OD reading of the eluted peak was calculated to be 3, which is equivalent to ~120 μg/ml of RNA, and approximately 480 μg/ml of protein in mRNP particles. The next step was to bind the associated protein kinase from the A+ fraction to a heparin column. A 1 ml heparin-Sepharose column (Pharmacia) was pre-washed with column binding buffer (20mM Tris (pH 8), 1mM EDTA, 1mM DTT, and 50mM KCl) and the A+ fraction was then loaded, after being adjusted to the binding buffer solution. The EDTA was included to destabilise the mRNP components, thus facilitating the disassociation of the associate protein kinase from the rest of the particle. The A+ fraction was cycled through the column multiple times, after which the unbound material was removed, and the column was washed with binding buffer until the absorbance trace returned to the base line (fig. 48). Bound protein was eluted using increasing concentrations of NaCl in 1ml aliquots of column binding buffer. The salt concentrations used were 0.05, 0.1, 0.2, 0.3, 0.4, 3 x 0.5, 3 x 1 and 1.5 M NaCl. The bound protein kinase was expected to elute at around 1 M NaCl (John Sommerville unpublished). The column was therefore washed 3 times with 0.5 M NaCl to ensure most of the

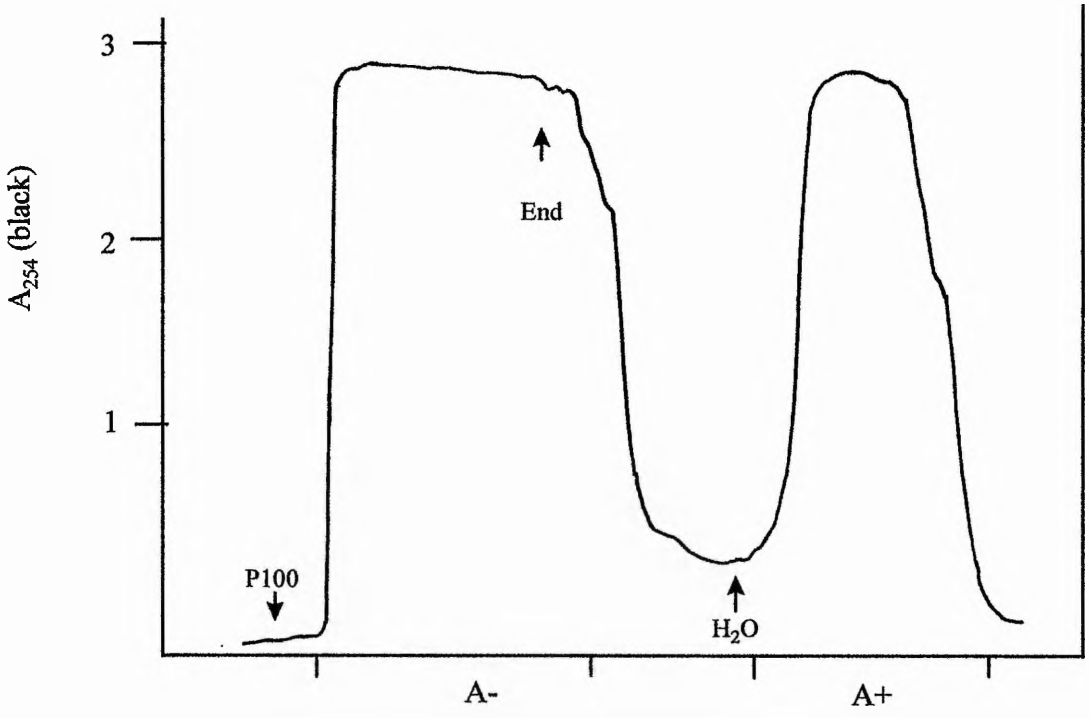



**Figure 47: Elution of poly(A+) mRNP from an oligo (dT) Sepharose column:** Elution of mRNP was measured via OD readings at $A_{254}$ using a quartz 3mm path-length cell. Oocyte extract was loaded onto the column (**P100**) and recycled three times. The unbound material was collected (**End**) and the column was washed until it returned back to baseline. Warm $H_2O$ was added to elute the poly (A+) mRNP ($H_2O$), producing a peak of OD 3. The A+ was collected in 3 ml of $dH_2O$.

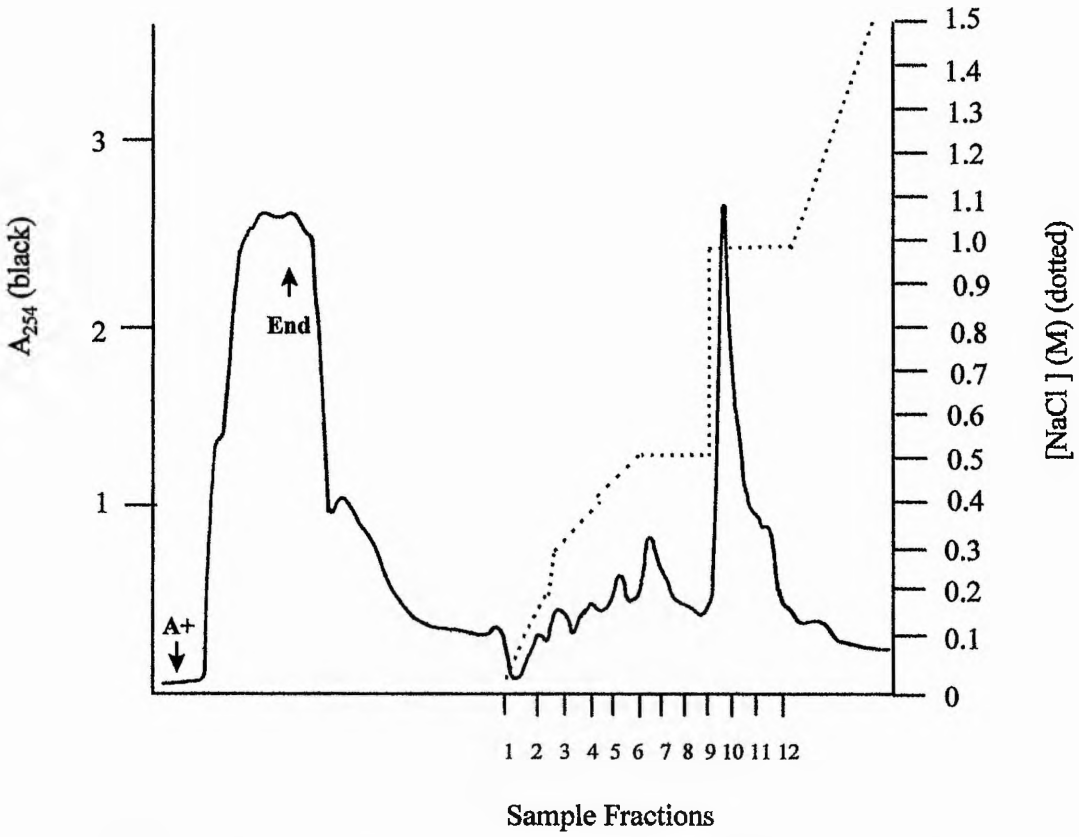



**Figure 48: Elution of mRNP-associated protein kinase from an heparin-Sepharose column**: Elution of RNA and proteins was measured via OD readings at $A_{254}$. Poly (A+) mRNP was loaded onto the column (**A+**) and recycled three times. The unbound material was collected (**End**) and the column was washed until it returned back to the baseline. Increasing NaCl concentrations were added to elute bound protein (0.05-1.5M) and were collected in 1ml fractions labelled 1-12. The largest peak, representing the highest concentration of eluted proteins, was seen in fraction 9 at the first 1M NaCl elution. It produced a peak of OD 2.6.

weakly-bound proteins were eluted and 3 times with 1 M NaCl to ensure all the protein kinase activity was eluted. The absorbance after each salt elution was recorded in order to determine in what fraction the protein kinase might be eluting from the column (fig. 48). 1 ml of each elution step was collected off the column and placed on ice, or frozen for later use.

The absorbance trace of salt elutions (fig. 48) shows that the largest peak of eluted proteins was seen during the 1 M NaCl wash. Other smaller peaks can be seen before this elution step, representing more weakly bound proteins. The first large peak represents the bulk of the unbound mRNP, probably including most of the poly (A)+ RNA.

*5.3.2 Activity of partially purified mRNP protein kinase*: The activity of the eluted protein kinase from mRNP was measured by phosphorylation of a known CK2 substrate (GST-HDΔV) in the presence of [$^{32}$P]γ-ATP. 10 μl of each of the salt elutions was incubated in kinase buffer (20 mM Tris (pH 7.4), 20mM $MgCl_2$, 1mM DTT and 100mM KCl) with 1μl of GST-HDΔV (~20μg) and 0.03 mM of [$^{32}$P]γ-ATP in a final volume of 100μl. Samples were incubated at room temperature for 1 hr with gentle agitation. The samples were then loaded onto a protein gel which was then stained with Coomassie brilliant blue, destained, dried down onto 3M paper using a vacuum drier, and set up for autoradiography (see Methods, section 2.6). This same gel was then scanned using an instant imager to record the exact counts in each track which were then graphed (see Methods, section 2.15).

The results of this assay can be seen in figure 49 which shows the stained protein gel, the autoradiograph and the graph of the incorporated label. The stained gel showed (fig. 49A) that equal amounts of GST-HDΔV had been added

to each track, and that although the protein eluted from the column has been added to each sample, the amount is too small to be visualised as discrete protein bands on this gel. The phosphorylation of GST-HDΔV can be seen in the autoradiograph (fig. 49B), but only in the 1 M NaCl, 1.5 M NaCl and unbound fractions. This phosphorylation pattern indicates that the protein kinase has eluted mainly in the 1 M NaCl wash, and to a smaller extent in the 1.5 M NaCl. Also it illustrates that there is still protein kinase activity in the unbound fraction, but this is to be expected since the heparin column is probably saturated and cannot bind all the A+ proteins. The majority of the incorporated counts are in the GST-HDΔV substrate which has been phosphorylated with the protein kinase activity from the 1 M NaCl wash (fig. 49C).

*5.3.3 Identification of mRNP-associated protein kinase*: Immunoblots of the salt washes from the heparin column were analysed in an attempt to verify that there was indeed CK2 present in those elutions. Since the highest protein kinase activity was observed in the 1 M NaCl elutions, these samples and the samples directly preceding and following the 1M elution were blotted with anti-CK2α. A 200µl sample was taken from each elution fraction and were precipitated in four volumes of acetone. The samples were left on ice for 30 min after which they were spun at 10,000 rpm for 20min. The supernatant was removed and the precipitated proteins were dried down in a vacuum oven for 10 min. These pellets were raised in 20µl of SDS-PAGE buffer and loaded on an SDS-PAGE gel (see Methods, section 2.6). Protein was transferred from the gel

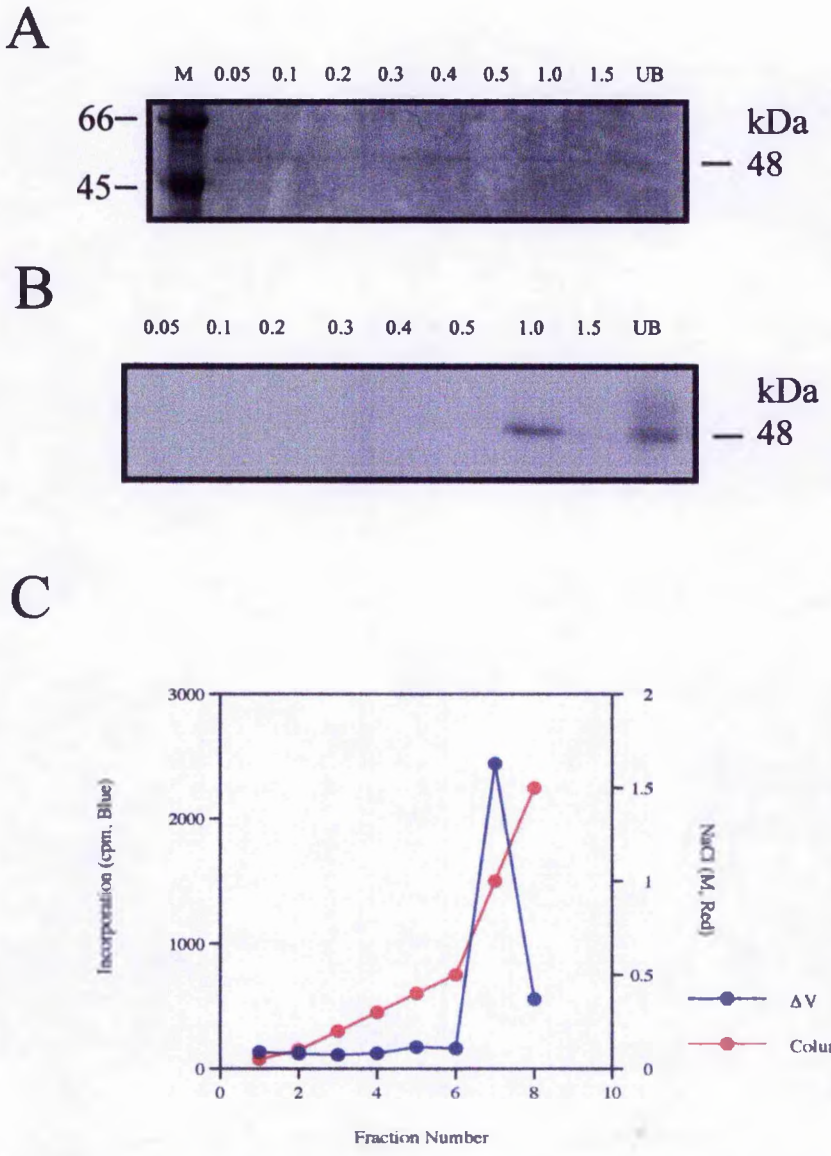



**Figure 49: Protein kinase activity assay of heparin-Sepharose column fractions**: A) Coomassie stained protein gel of the phosphorylation assay showing GST-HDΔV (48 kDa) at equal concentration across the assay. GST-HDΔV is the only protein concentrated enough to be visualised on this gel. Markers at 66 and 45 kDa are also shown. B) Autoradiograph of [$^{32}$P]γ-ATP labelled GST-HDΔV catalysed by elutions from the heparin column, ranging from 0.05M-1.5M NaCl and the unbound fraction (UB). GST-HDΔV (48 kDa) is predominately phosphorylated by the 1M NaCl elution and the unbound fraction and to a lesser extent by the 1.5M elution. C) Graph depicting the total counts in GST-HDΔV phosphorylated by each elution fraction. The blue line represents the extent of phosphorylation of GST-HDΔV in each track, the highest counts being recorded in fraction 7 which in this figure represents the 1M NaCl elution. The red line represents the increasing NaCl concentration used to elute each fraction.

to nitrocellulose paper and immnoblotted with anti-CK2α (see Methods, section 2.7). After detecting the antigen using ECL, the transfer was stained with amido black (1.5g of amido black, 45 ml of methanol, 10ml of glacial acetic acid, and up to 100 ml with $dH_2O$), for 15 min with gentle agitation and then destained (180 ml of methanol, 4 ml of glacial acetic acid, adjusted to 200 ml with $dH_2O$), for 15 min with gentle agitation.

Figure 50A and B shows that the two CK2 α-subunits (42 and 38 kDa) are present in the highest concentration in 1A, which represents the first 1M NaCl elution fraction from the heparin column. This same fraction is also shown to have the highest activity of all the 1M NaCl elutions (fig. 50C). The remaining 1M elutions: B and C show little immunoreactivity (fig. 50B), and correspondingly less protein kinase activity in their inability to phosphorylate GST-HDΔV (fig. 50C). The 0.5 M and 1.5 M elutions, show negligible immunoreactivity for CK2α (fig. 50B) and also negligible phosphorylation activity of GST-HDΔV (fig. 50C). Stage VI nuclei (~10) were loaded on this gel as a positive control for anti-CK2α, which shows immunoreactivity for CK2α at identical positions (molecular weights) for each of the two α-subunits.

*5.3.4 Inhibitors of mRNP-associated protein kinase*: In order to verify that the CK2 associated with mRNP was similar in most respects to that CK2 found in the nucleus, activity assays were preformed in order to test the sensitivity of this protein kinase to well established inhibitors of nuclear CK2 activity. Phosphorylation of the substrate GST-HDΔV was used to test the inhibitory

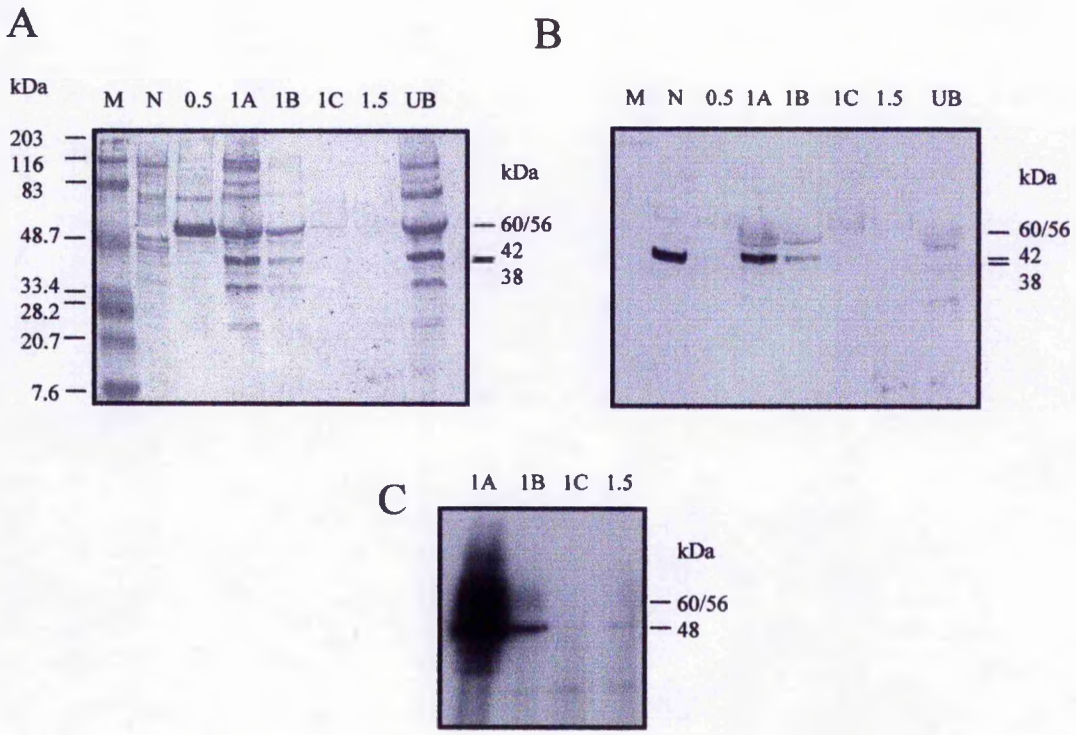



**Figure 50: Identification of the protein kinase activity eluted from the heparin-Sepharose column**: A) Nitrocellulose transfer stained with amido black showing the protein content of the immunoblotted samples. The main bands of interest are the Y-box proteins (60 and 56 kDa) and the CK2 α-subunits (42 and 38 kDa). Prestained markers are present in the first track. B) The ECL immunoblot of (A) with anti-CK2α. The immunoblot shows that CK2α is most abundant in fraction 1A, with some lesser signal in 1B. The N track is stage VI nuclei added as a positive control. C) Phosphorylation of GST-HDΔV with protein kinase purified with 1 M NaCl (1A, 1B, 1C) and with 1.5 M NaCl using [$^{32}$P]γ-ATP. The highest kinase activity is seen in the 1A fraction with a lesser amount also seen in the 1B fraction.

affects of the various established CK2 inhibitors (quercetin, DRB and heparin) on the mRNP-associated protein kinase. The phosphorylation assays were 10μl reactions which consisted of: 2μl of the eluted 1 M NaCl fraction (containing the mRNP-associated protein kinase), 1μl of [$^{32}$P]γ-ATP (0.03mM final), 1μl of 10 x kinase buffer (final: 20mM Tris (pH.7.4), 20mM $MgCl_2$, 1mM DTT, and no NaCl), 2μl of the protein substrate GST-HDΔV(~1μg), 1μl of various diluted inhibitors and 3μl of $dH_20$. These reactions were left to incubate at room temperature for ~1 hr, after which 10μl of SDS-PAGE buffer was added to each reaction and the samples were loaded onto an SDS-PAGE protein gel. Gels were stained with Coomassie blue, destained, dried onto 3M paper and setup for autoradiography (see Methods, section 2.6).

The results from the assay can be seen in figure 51 and show that the known CK2 inhibitors all have a significant inhibitory effects on the phosphorylation of GST-HDΔV (48 kDa) and on the endogenous Y-box proteins (60 and 56 kDa) by the mRNP-associated protein kinase. The first track called (C) is a control representing the phosphorylation of the substrate protein with no addition of protein kinase inhibitors. The following tracks all represent addition of inhibitors to the phosphorylation reaction. Track (Q) represents the phosphorylation with 20 ng/ml of quercetin added; this shows considerable inhibition of CK2 phosphorylation. The next track (R) is the addition of rutin, the inactive analogue of quercetin, used here as a negative control. The addition of 20ng/ml of rutin fails to inhibit phosphorylation of GST-HDΔV by CK2. The addition of DRB seen in the D) track also shows efficient inhibition of protein kinase activity, thus showing the sensitivity of this eluted protein kinase to the

CK2 inhibitor. The next three tracks represent a serial dilution of the inhibitor heparin. The additions are 100, 10, and 1 μg/ml of heparin in respective tracks. The sensitivity of the protein kinase is evident in the higher concentration of heparin added (100 and 10 μg/ml), but also some inhibition of the protein kinase is observed at the lowest concentration of 1μg/ml. Thus it can be seen from this result that the protein kinase associate with mRNP is sensitive to all the known inhibitors of CK2 and at similar concentrations. This provides compelling evidence that the protein kinase eluted from the mRNP is indeed CK2.

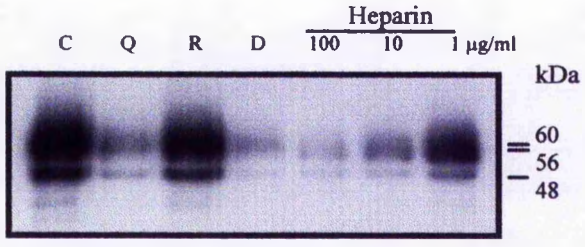



**Figure 51: Phosphorylation of GST-HDΔV by mRNP-associated protein kinase: effects of various inhibitors**: The C) track is a control phosphorylation of the substrate GST-HDΔV (48 kDa) by kinase from mRNP, using [$^{32}$P]γ-ATP as a label. Phosphorylation of eluted Y-box proteins (60 and 56 kDa) can also be seen. Q) Same as in C) except that 20 ng/ml of the inhibitor quercetin added. R) The same as in C) except that 20 ng/ml of rutin added. D) Same as in C) except that 50mM/ml of the inhibitor DRB added. 100) Same as in C) except that 100 μg/ml of the inhibitor heparin added. 10) Same as in C) except that 10 μg/ml of the inhibitor heparin added. 1) Same as in C) except that 1 μg/ml of the inhibitor heparin added.

## 5.4 Sequence of the Y-box Proteins Phosphorylated with CK2

*5.4.1 Sequence of Y-box proteins phosphorylated by nuclear CK2*: Y-box proteins were phosphorylated using stage VI nuclei and [$^{32}$P]γ-ATP in order identify the sites phosphorylated by this source of CK2 activity. Y-box proteins were purified from immature oocytes using a method of successive heat

treatments in order to precipitate contaminating proteins (see Methods, section 2.18.2). This substrate was then treated with alkaline phosphatase for 1 hr at 37°C in CIP buffer (100mM Tris (pH. 8.3), 10mM $MgCl_2$, and 10mM $ZnCl_2$) to remove any pre-existing phosphates from potential sites. The phosphatase was inactivated and effectively removed by successive heat treatments (see Methods, section 2.18.2). The Y-box proteins were added to a phosphorylation reaction containing stage VI nuclei and [$^{32}$P]γ-ATP. The phosphorylation reactions were 100µl reactions which consisted of: 10µl of stage VI nuclear kinase (equivalent to 10 nuclei), 10µl of [$^{32}$P]γ-ATP (0.03mM final), 10µl of 10 x kinase buffer (final: 20mM Tris (pH.7.4), 10mM $MgCl_2$, 1mM DTT, and 100mM KCl), 20µl of the protein substrate Y-box (~5µg), and 50µl of $dH_20$. These reactions were left to incubate at room temperature for 1 hr. The samples were then acetone precipitated using 4 volumes of acetone and the pellets were dried down in a vacuum oven. A portion of the pellet was raised in 8M urea and SDS-PAGE buffer after which 10µl the sample was loaded onto an SDS-PAGE protein gel. Gels were stained with Coomassie blue, destained, dried onto 3M paper and setup for autoradiography (see Methods section 2.6). The rest of the sample was given to Dr. Graham Kemp in order to sequence labelled peptides from trypsin digests of the proteins and to identify the phosphorylated residues. The protein gel and autoradiograph were presented to him to aid in identification of phosphorylated proteins. Sequencing and identification of the labelled residues were preformed using the procedure written in the Methods section 2.12.

The sequencing of the Y-box proteins has not been completed, but preliminary results indicate that only some of the phosphorylation sites are

labelled by nuclear CK2. The FRGY2a/b protein was digested with trypsin and separated by fine-bore HPLC. Labelled peptides were sequenced to determine which residues were phosphorylated. Of the several small peptides recovered only one was sequenced accurately. Other short peptides either did not bind to the hydrophilic column or precipitated out of solution. The sequence shown in figure 52 indicates that of the three possible phosphorylation sites only one, the serine at position 155, seems to be labelled. Since these are only preliminary results not much can be stated about this finding. The possibility that this site is necessary for the binding of FRGY2a/b to mRNA is unknown. This information does show however, that only some of the potential target sites are used by nuclear CK2, leaving other sites free for possible phosphorylation by CK2 in the cytoplasm or at a later stage of development.

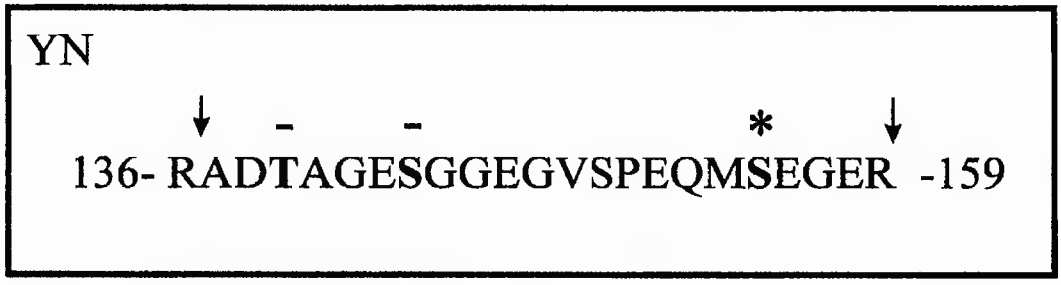



**Figure 52: Sequence of FRGY2 showing phosphorylated residues**: FRGY2 phosphorylated by nuclear CK2 (YN). The sequence shows that the first two potential sites (bold) are not phosphorylated (-) but the third site may be (*). Arrows indicate the sites of trypsin digestion.

*5.4.2 Sequence of Y-box proteins phosphorylated by mRNP-associated CK2*: Y-box proteins bound to mRNA were phosphorylated using native mRNP particles in order to determine if the residues phosphorylated by the associated CK2 were the same as those phosphorylated by the CK2 from nuclei. The mRNP

was purified from homogenated oocyte extracts using an oligo(dT) column and eluted with ~ 1ml $dH_2O$ (see Methods, section 2.18.1) The clarified homogenate was pre-incubated with alkaline phosphatase prior to the column step, to remove any pre-existing phosphates. The de-phosphorylated poly(A)+ mRNP was then incubated with 0.03mM [$^{32}$P]γ-ATP at room temperature for 1 hr. The rest of the procedure is identical to the previous section.

No data on the phosphorylation state of FRGY2a/b, by mRNA-associated protein kinase, is available at this time.

**5.5 Summary**

The Y-box proteins, specifically the *Xenopus* oocyte FRGY2a/b, of 60 and 56 kDa have several potential phosphorylation sites for the protein kinase CK2. In order to determine if these proteins were in fact phosphorylated by CK2, a number of assays were performed to describe the activity of the phosphorylating protein kinase. The Y-box proteins were phosphorylated by the oocyte nuclear protein kinase activity described in Chapter 3, under varying conditions of added salts, ions and kinase inhibitors. The phosphorylation conditions detected were very similar to the conditions that define CK2 activity (reviewed in Allende and Allende, 1995). The phosphorylation is sensitive to DRB, quercetin, heparin, salt concentration, $Mg^{2+}$ concentration and the competitive inhibitor poly(EY).

*In vivo,* the Y-box proteins are found associated with mRNP and it is thought that their association with mRNA is facilitated by phosphorylation (Sommerville, 1992). There is also an associated protein kinase found within these mRNP particles which is thought to be CK2 (Dearsly *et al.* 1985; Kick *et*

*al.*, 1987; Cummings *and* Sommerville, 1988; Braddock *et al.* 1994). In this study, the kinase was found to bind to heparin-Sepharose columns, and to be eluted in 1M NaCl, retaining its activity. Y-box proteins and GST-HDΔV protein were used as substrates in order to determine which elution fractions contained protein kinase activity, and also to confirm that this activity did indeed phosphorylate these two substrates. The fractions were also immunoblotted with anti-CK2α, which showed a strong signal of the appropriate size (42, 38 kDa) in the most active fraction, thus providing strong evidence that CK2 is indeed the associated kinase that phosphorylates the Y-box proteins. The protein kinase associated with mRNP was further purified and tested against the known inhibitors of CK2. The sensitivity of this protein kinase to DRB, quercetin, heparin, salt concentrations, $Mg^{2+}$ concentrations and the competitive inhibitor poly(EY) was consistent with the activity defined in earlier work (reviewed in Allende and Allende, 1995) and to CK2 activity found in assays done in previous chapters of this thesis (Chapters 3 and 5).

The sequence of the Y-box proteins, after phosphorylation with the nuclear and mRNP-associated CK2 kinase, was begun in order to investigate which sites were used for nuclear and for cytoplasmic phosphorylation. Once these sites are identified the function of each of the phosphorylated sites could be studied by site-directed mutagenesis. This experiment is still ongoing, but preliminary results indicate that only some of the potential sites are phosphorylated in the nucleus, possibly leaving other sites for cytoplasmic phosphorylation later in development.

# Chapter 6: Discussion

### 6.1 Nuclear Activity of CK2

The protein kinase CK2 has recently been extensively studied in embryonic and proliferating cells, which prompted the study in oocytes, since oogenesis represents one of the initial stages of development. The results reported illustrate that the level of CK2 steadily increases during oogenesis, and that its highest concentrations appear in the nucleus (fig. 16). The observation that it exists as a ~130 kDa particle seems to indicate that it is predominantly not part of a large protein complex, since ~130 kDa is the expected size of the tetrameric protein (fig. 18). CK2 may form only transient complexes with other proteins when enzymatically active. The fact that nuclei isolated from stage VI *Xenopus* oocytes supply such a rich source of CK2 activity would indicate that CK2 is an important protein kinase during this stage of development. The activity isolated from stage VI nuclei fits all the criteria known to distinguish CK2 activity: use of ATP and GTP as co-substrates, inhibition by heparin, DRB, quercetin, stimulation by spermine, and sensitivity to monovalent and divalent cations. The fact that CK2 has so many possible substrates, and seems to participate in so many diverse processes, suggests at least some involvement in early development (Allende and Allende, 1995). There are many possible functions that CK2 can preform in the nucleus, and it has indeed been implicated in many nuclear mechanisms. It is shown, in this report, that nuclear CK2 is found in high concentrations around the nuclear envelope, and in the nucleoli (fig. 17). It is also thought to be associated with the nuclear matrix (Tawfic *et al.*, 1996). Given these observations, CK2 has the potential to be involved in numerous nuclear mechanisms involving nuclear proteins such as: histone deacetylase, nucleolin and nucleoplasmin. Nuclear CK2 could be involved in

transcription activation (Sommerville *et al.*, 1998), RNA synthesis (Pfaff *et al.*, 1988; Bouch *et al.*, 1994), signal transduction (Csermely *et al.*, 1993) and nuclear import (Vacurova *et al.*, 1995).

*6.1.1 Regulation of a maternal histone deacetylase*: One possible nuclear role of CK2, investigated in this thesis, was the regulation of HDACm activity by phosphorylation. HDACm has several potential CK2 phosphorylation sites (fig. 29, 34, 35, 53), and is shown to exists in different phosphorylation states in the oocytes (Llinas *et al.,* in preparation). HDACm has been immunoblotted from nuclear extracts and shows that HDACm exists as multiple bands, possibly representing the varied phosphorylation states of this protein. These bands were resolved to a single immunoreacting band when the extracts were pretreated with alkaline phosphatase (Llinas *et al.*, in preparation). Immunoreactive HDACm bands have been observed migrating slower and probably represented phosphorylated forms from extracts of embryos at midblastula (Llinas *et al.*, in preparation). This observation is similar to the changes seen in *Xenopus* nucleoplasmin migration reported to occur just after the midblastula transition when nucleoplasmin is dephosphorylated (Burglin *et al.*, 1987). Results have shown that treatment of extracts containing the 360 kDa histone deacetylase complex with alkaline phosphatase leads to a loss of 60-90% of enzyme activity (fig. 32). Other forms of histone deacetylase have been shown to lose substrate specificity when treated with alkaline phosphatase (Brosch *et al.*, 1992). The phosphorylation state could provide an important control mechanism in preventing the premature deacetylation of accumulating histones that takes place during oocyte development. The core histones accumulate in acetylated forms in order to be incorporated with newly synthesised DNA, after fertilization, to form

nucleosomes. The histone deacetylase activity has been shown to be high in stage VI nuclei (fig. 33), so a nuclear regulatory mechanism for this enzyme is necessary and could involve phosphorylation by CK2. There are other potential kinase sites within the HDACm protein, such as: protein kinase C, tyrosine kinase and cyclic AMP-dependent kinase sites (fig. 53), which may be involved in regulating activity, but there is, so far, no data to suggest the involvement of protein kinases, other than CK2.

*6.1.2 Regulation of pre-mRNP*: Another possible nuclear function of CK2, investigated in this thesis, is the regulatory role of CK2 on the masking of mRNA by Y-box proteins. Past research has discussed a protein kinase activity associated with mRNP, but the identity of the kinase, and what purpose it served was not determined (Cummings *et al.*, 1988). The masking of mRNA is reported to be dependent on the position of introns within the pre-mRNA. It is thought that introns, positioned at the 5' end of the message, block the ability of mRNA to be masked, but introns located at the 3' end do not interfere with this masking process (Matsumoto *et al.*, 1998). It could be that the spliceosomes involved in removal of these introns might interfere with the binding of masking proteins, but the mechanism is still unknown. The position of these introns seems to be a method of controlling whether certain messages are masked and thus stored, or whether they are actively translated. CK2 is unlikely to take part in this splicing mechanism, but earlier studies have shown that the binding of the masking proteins is affected by phosphorylation (Braddock *et al.*, 1994). This report showed that the kinase "protein kinase CK2" is associated with mRNP particles and that its activity is responsible for the phosphorylation of the two associated Y-box proteins FRGY2a and FRGY2b (fig. 50). The mechanism involving this

kinase is still unclear, but microinjection experiments have shown that nuclear phosphorylation of the YB proteins is essential for mRNA masking to occur (Braddock *et al.,* 1994). It could be that the phosphorylation state of the Y-box proteins could have a direct effect on their ability to bind mRNA. Other work has complemented these findings by showing that mRNA masking is initiated in the cell nucleus (Bouvet and Wolffe, 1994), but the role of phosphorylation is still debated. Further information on a nuclear role comes from oocyte microinjections experiments which showed that masking could be reversed by the co-injection in the nucleus of anti-pp60 and anti-p54 IgG with reporter mRNA constructs, but not by co-injection of control IgG (Braddock *et al.*, 1994). The point most relevant to this thesis is that messenger RNA masking was also blocked by delivering, into nuclei, either chemical inhibitors of casein kinase II, or IgG raised against chicken casein kinase II (CK2) subunits (Braddock *et al.*, 1994).

Another possible effect of CK2 phosphorylation, which was not investigated here, could be the regulation of the interaction between Y-box protein, or the mRNA itself, and a reporter protein designed to facilitate nuclear export, such as transportin (Wozniak *et al.*, 1998). Although no work has been done to show this relationship, it is thought that the nuclear import and export pathways might use similar mechanisms (Wozniak *et al.*, 1998). CK2 has indeed been implicated in the nuclear import pathway, examples being: SV40 T-antigen, nucleoplasmin and nucleolin (Rihs *et al.*, 1991; Vancurova *et al.*, 1995; Schwab and Dreyer, 1997).

## 6.2 Cytoplasmic Activity of CK2

The activity of CK2, in oocyte cytoplasms, is reportedly quite high (Wilhem *et al.*, 1995), so the possible functions of this compartmentalised enzyme must be elucidated . One possible function of CK2 in the cytoplasm, could relate to the compartmentalisation of the HDACm enzyme.

*6.2.1 Cytoplasmic retention of histone deacetylase*: Extracts taken from all stages of *Xenopus* oocyte development have histone deacetylase activity; this would seem to indicate that the separation of HDAC activity from its potential substrates (stored acetylated histones) might involve a form of compartmentalisation within the cells, instead of the regulated expression of inhibitory factors. Since it is quite difficult to extract, in a soluble form, all of the HDACm protein from early stage oocytes, this might suggest that there are structures responsible for the anchorage of HDACm in the cytoplasm which, in turn, might act in a regulatory role to limit the activity of this deacetylase. The HDACm antigen, from early stage oocytes (I-III), was recovered more efficiently when the organic solvent Freon was used, which solubilizes membranous material. When this solvent is not used, HDACm is lost in the pellet fraction after low speed centrifugation. This non-soluble, fast sedimenting HDACm fraction appears localised on observing immunostained sectioned oocytes. It is seen that a significant fraction of HDACm is associated with the oocyte membranes and particularly in vesicular structures close to the cell perimeter. It is possible that these sites represent structures responsible for the early anchorage of HDACm in the cytoplasm. This compartmentalisation of HDACm may be responsible for the lack of association of this protein with chromosomes.

Experiments have indicated that that lampbrush chromosomes isolated from nuclei of mid-stage oocytes are not associated with HDACm, but are shown after injection with excess HDACm to be associated with immunoreactive protein. Oocytes treated with sodium butyrate, an inhibitor of deacetylase activity, showed that hyper-acetylation of histone H4 in lampbrush chromosomes did not occur, indicating that acetylation of the chromatin was not in a dynamic state (Sommerville *et al.*, 1993). This suggests that the deacetylase activity is sequestered away from the chromosomes. Injection of excess amounts of recombinant HDACm into oocytes leads to a release from the constraints which might anchor the endogenous HDACm in the cytoplasm. Import of recombinant HDACm into the nucleus appears to lead to chromatin binding and eventually premature condensation of the chromosomes. This condensation is blocked by low concentrations of trichostatin A (TCA), specific inhibitor of HD activity. It would therefore seem important that the native histone deacetylase activity remains separate from the chromosomes by possible compartmentalisation in the cytoplasm. This sequestration of HDACm in the cytoplasm might be achieved by membrane attachment mediated by phosphorylation of the protein. Phosphorylation of proteins has been linked to cytoplasmic sequestering of normally nuclear proteins, such as nucleolin (Schwab and Dreyer, 1997), but most were proposed to be regulated by cell cycle kinases, such as cdc-2 kinase. Research involving the cytoplasmic anchoring of nuclear-imported proteins have shown that there is, usually associated near to the NLS, a cell-cycle dependent cdc2 kinase phosphorylation site, as is the case with large T-antigen (Jans, 1995). HDACm does not have such a site within its amino acid sequence, but other unknown kinase sites may function in similar ways. Also, a sequence has been

described that is thought to be specific for cytoplasmic retention, EEYYAHYVGLNRRQNEWVDKSR, which exists in the *Xenopus* protein nuclear factor 7 (xnf7). The protein xnf7 has been shown to be a cytoplasmic protein until it is dephosphorylation at mid-blastula, when it then becomes nuclear (Li *et al.*, 1994). Although this particular sequence is not present in HDACm, other alternative sequences serving the same function may exist: in fact other proteins retained in the cytoplasm lack the xnf7 sequence (Schwab and Dreyer, 1997). An alternative sequestering mechanism may involve the NLS of HDACm being somehow masked in the cytoplasm, thus preventing its nuclear import. The NLS of NF-κB has been shown to be masked by IκB, until IκB is phosphorylated by PKC which initiates IκB degradation (Diaz-Meco *et al.*, 1994). Although this could be a possible mechanism for the masking of HDACm, there has been no information to support this hypothesis. An alternative possibility is NLS masking by phosphorylation. Nuclear transport of lamin B2 has been shown to be inhibited by the phosphorylation of two PKC sites near the NLS (Hennekes *et al.*, 1993). There are multiple PKC sites in HDACm, one of which is quite close to the NLS (504-506, SVK), (fig. 53). Although, the PKC site near the NLS is not seen to be phosphorylated during nuclear import of the GST-HDΔV fusion protein, which could mean that it is dephosphorylated to allow nuclear import, there is no evidence for phosphorylation of PKC sites while HDACm is in the cytoplasm and so no firm conclusion can be drawn.

*6.2.2 Nuclear import of histone deacetylase*: Another cytoplasmic role for CK2 is the enhancement of nuclear import, which has been widely studied with many diverse proteins (Rihs *et al.*, 1991; Jans and Jans, 1994; Vancurova *et al.*,

1995; Schwab and Dreyer, 1997). Observations made in this thesis suggest that phosphorylation by a cytoplasmic form of CK2 enhances the kinetics of nuclear import of HDACm. Studies were carried out using the fusion protein GST-HDΔV. This fusion protein is phosphorylated at multiple sites by CK2 and also has a bipartite nuclear localization signal at its carboxyl tail domain (fig. 34). It is thought that the function of the NLS can be regulated by phosphorylation of specific sites, which may either accelerate or inhibit nuclear import. The nuclear import of proteins such as nucleolin, nucleoplasmin and SV40 T-antigen have been shown to be increased in some cases as much as 40 fold by phosphorylation of specific CK2 sites (Rihs *et al.*, 1991). Serine$^{111/112}$ are examples of such a sites where this occurs. This phosphorylation may regulate protein-protein interaction between the NLS and nuclear import proteins. The sequence that has been identified as a possible NLS in HDACm consists of residues 438-**KK**V**KR**V**K**TEEEKEGED**KK**DV**K**-458 existing within the carboxyl-terminal region of this protein. The injection experiments described in this thesis show that fusion proteins missing this region of the carboxyl tail domain are imported into the nucleus less efficiently (fig. 37). The NLS of HDACm is similar to the one described for the *Xenopus* protein N1/N2, which consists of residues 531-LV**RKKRK**TEEESPLKD**K**DA**KK**-551 (Dingwall and Laskey, 1991). The function of HDACm and N1/N2 are directly related to each other in that they both function with histones during early development of *Xenopus*. N1/N2 acts as a chaperone for acetylated histones H3/H4 (Kleinschmidt *et al.*, 1985) which are incorporated into chromatin, after which they are deacetylated by histone

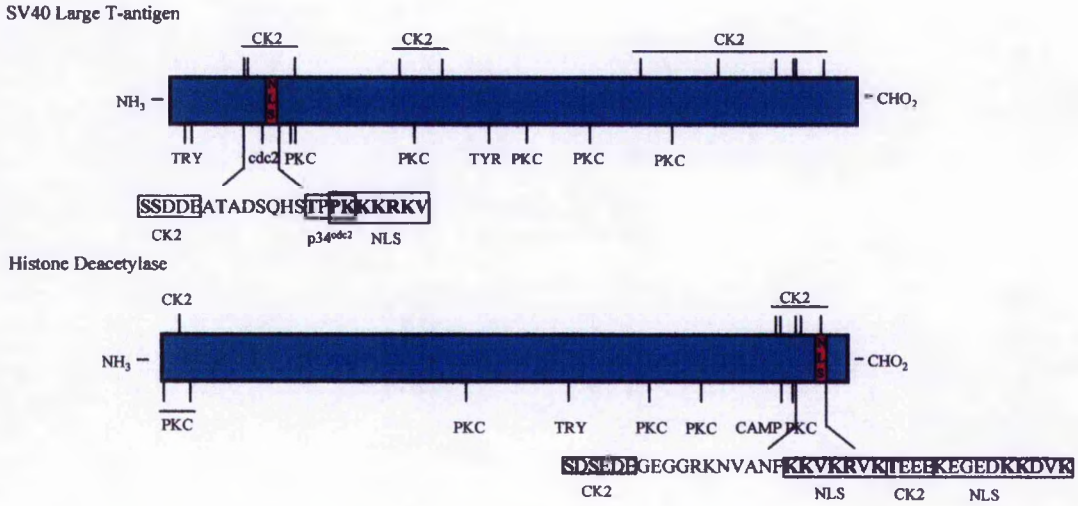



**Figure 53: Phosphorylation sites in large T-antigen and histone deacetylase**: Potential phosphorylation sites in SV40 Large T-antigen and histone deacetylase (HDACm). PKC= Protein kinase C sites, TRY= Tyrosine kinase sites, CK2= Protein kinase CK2 sites, $p34^{cdc2}$= cdc-2 kinase (MFP) sites, CAMP= Cyclic AMP dependent kinase sites. Positions of CK2 sites are shown in relation to the nuclear localisation signal (NLS) for both HDACm and Large T-antigen.

deacetylase to form new nucleosomes (Wade *et al.*, 1997). Thus, it seems feasible that these two proteins may be regulated in a similar manner. The specific sites in HDACm that were shown to be phosphorylated were at -15, -17 and -45 (fig. 36) from the start of the NLS in GST-HDΔV. The location of these sites are similar to the ones described for SV40 T-antigen, which were the CK2 sites at -14 and -15 from the NLS and were shown to enhance the rate of nuclear import (Rihs *et al.*, 1991; Jans and Jans, 1994). The CK2 site in GST-HDΔV fusion protein that was observed as not being phosphorylated was 145-TEEE-148, which lies within the NLS in both HDACm and in the protein N1/N2. Although it was not observe as being phosphorylated, it maybe phosphorylated under different conditions not met by the phosphorylation assay described here. The mechanism of CK2-mediated enhanced nuclear import has always been unclear, even for well-studied proteins such as large T-antigen. It has been thought that the mechanism operates by accelerated or facilitated interaction with the NLS binding proteins, or by enhanced docking with the nuclear pore complex (NPC), (Jans, 1995). An attempt was made to investigate this relationship in this project. One of the possible functions of CK2 phosphorylation, in relation to nuclear import, was the relationship between the phosphorylation state of HDACm and binding of the transport protein α-importin. The results presented indicate that binding of α-importin to the NLS is influenced by the phosphorylation state of the carboxyl tail domain of the HDACm fusion protein GST-HDΔV (fig. 40, 41, 42). Speculation has existed regarding the mechanism of dissociation once α-importin has been transported, with the substrate, into the nucleus. It was suggested that since there is such a high concentration of NLSs

in the nucleus, that α-importin must be converted into a form with low affinity for the NLS (Gorlich and Mattaj, 1996). It is suggested here that the phosphorylation of specific residues, in or close to the NLS, might disrupt this protein-protein interaction. Other processes that inactivate binding by α-importin might also occur simultaneously, in order to facilitate the release of cargo and to enhance the export of the α-importin from the nucleus. The association of α-importin with NLSs has been shown to be dependent on the presence of Ran-GTP, but not Ran-GDP (Kutay *et al.*, 1997). This is consistent with the observation that Ran-GTP is necessary for export (Moroianu *et al.*, 1997) and that Ran-GTP interrupts the α-β importin interaction (Rexach and Blobel, 1995). It is likely that Ran-GTP is predominant in the nucleus and Ran-GDP is predominant in the cytoplasm, thus creating a gradient that seems purposeful in its shuttling of α-importin (Wozniack *et al.*, 1998). This high concentration of GTP in the nucleus might also point to a parallel activity for the phosphorylation induced disassociation studied here, since CK2 can use GTP efficiently as a co-substrate. Although ATP is present at higher concentration in the nucleus than GTP, and there seems to be a greater disassociation effect on GST-HDΔV and α-importin with ATP (fig. 43), it might be important that CK2 be able to use both co-substrates, or be at least sensitive to the levels of each as a potential regulatory mechanism.

CK2 was shown (fig. 39) to facilitate nuclear import of the GST-HDΔV fusion protein, after injection into the cytoplasm. So how does this finding relate to the observations made with α-importin? One possibility is that cytoplasmic CK2 is part of a shuttling mechanism at the entrance to the NPC and its activity

is necessary for the initial steps of transport across the membrane. CK2 has been shown to be highly concentrated around the nuclear envelope (fig. 17). The envelope-associated CK2 could phosphorylate GST-HDΔV in the region of the nuclear envelope, thus facilitating the disassociation of α-importin from GST-HDΔV as they pass through the pore complex (fig. 54). Another possibility is that phosphorylation by cytoplasmic CK2 is necessary for efficient protein-protein interaction with an alternative shuttling protein. This phosphorylation could take place at an alternative residue from the one required for release of α-importin, which may require conditions not met by the assays preformed. The TEEE phosphorylation site within the NLS was shown to be unphosphorylated in our assays using CK2 activity obtained from isolated nuclei (fig. 36). However, this site may be phosphorylated under different conditions, requiring specific regulatory units which were not present during our tests.

*6.2.3 Unmasking of maternal mRNP*: The other cytoplasmic function of CK2 relevant to this discussion is a possible role in unmasking of mRNP particles in response to cell signaling. It is known that in response to cellular signals, such as progesterone induced maturation, certain kinases (MPF-kinases) are activated, promoting specific signaling pathways. CK2 does contain a $p34^{cdc2}$ kinase site, $p34^{cdc2}$ being one of the major MPF kinases, which makes CK2 involvement in this pathway possible. Also, results presented by Mulner-Lorillon *et al.* (1990) and Litchfield *et al.* (1992) illustrate that $p34^{cdc2}$ did indeed phosphorylate CK2 and led to its increased cytoplasmic activity. It is theorised that as a result of this progesterone induced activity, phosphatases are activated which might be responsible for dephosphorylating the Y-box proteins leading to

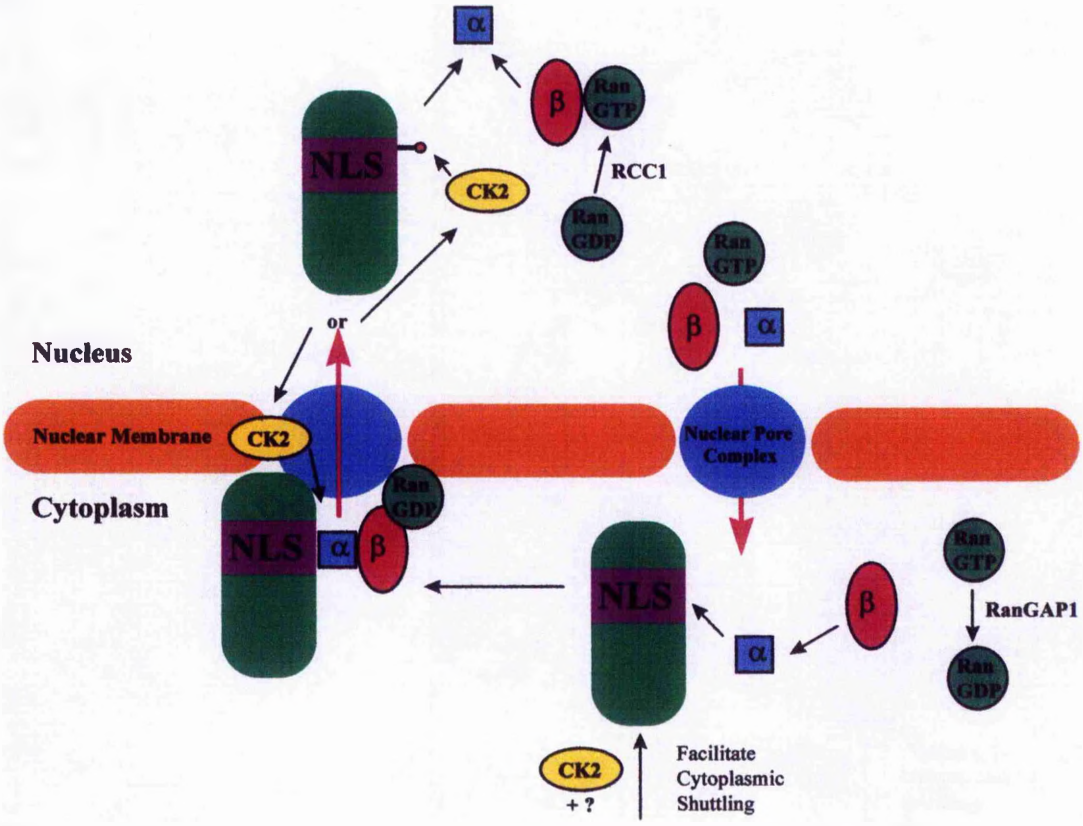



**Figure 54: CK2 promotes release of α-importin from NLS**: Possible roles of phosphorylation by CK2. CK2 facilitates nuclear import by phosphorylation of the NLS leading to binding of transport proteins. The phosphorylation of the NLS could alter the binding of α-importin as proteins pass through the nuclear pore complex or once inside the nucleus. The individual importin subunits then exit through the NPC and bind to other proteins to be transported.

the translation of the stored mRNA (Kick *et al.* 1987), (fig. 55). Kick *et al.* (1987) have shown that reconstituted FRGY2a:globin mRNA complexes were translationally inactive in wheat germ lysate, but became translationally active after dephosphorylation. This work was confirmed using the rabbit reticulocyte mRNP protein p50 (homologue of FRGY1) which was also shown to be phosphorylated, *in vivo* and *in vitro*. On reconstituting phosphorylated p50 with globin mRNA, the mRNA was rendered translationally inactive in wheat germ lysate: dephosphorylation led to translation activation (Minich and Ovchinnikov, 1992). Other work reported that treating *Xenopus* oocytes with progesterone reversed masking, and resulted in the translation of the previously masked reporter constructs (Braddock *et al.*, 1994). Progesterone acts, *in vivo,* to promote the maturation of oocytes, which involves the recruitment of various maternal mRNAs for translation (maternal mRNA recruitment starts at oocyte maturation and continues throughout fertilization and early embryogenesis). The progesterone-induced unmasking process has been blocked by okadaic acid, an inhibitor of phosphatase, in a dose-dependent response (Braddock *et al.* 1994). This is in agreement with the findings that the phosphorylation of YB proteins is necessary to maintain mRNPs translationally repressed (Kick *et al.*, 1987). Although phosphorylation of FRGY2 by CK2 may have little effect on the binding of FRGY2 to RNA *in vitro* (Deschamps *et al.*, 1997), it does lead to more stable protein-protein interactions within the mRNP particles (Ladomery and Sommerville, 1994). It has been reported that the activity of CK2 towards some substances is enhanced during progesterone-induced maturation (Cicirelli *et al.*, 1988; Kandror *et al.*, 1989), thus providing a convenient signalling pathway for unmasking by CK2. The phosphorylation by cdc2 kinase might

change the substrate specificity of CK2, thus preventing re-phosphorylation of the Y-box proteins once they have been dephosphorylated by progesterone-induced phosphatase. On the other hand, it may also induce CK2 to phosphorylate the Y-box proteins at alternative sites which aid in disrupting the protein-mRNA interaction. Or, it might also help regulate the specificity of the phosphatase activity towards the Y-box proteins, but data confirming either of these possibilities is not available.

The phosphorylation of proteins designed to initiate the unmasking of mRNAs is another possible function for the associated CK2. A possible mechanism of unmasking is the association of masked mRNA with the nuclear protein nucleoplasmin (Meric *et al.* 1997). Nucleoplasmin is an abundant nuclear chaperone protein, which has been cross linked to mRNA in the cytoplasm during the unmasking process. It is thought that once the oocyte nucleus has broken down, after maturation with progesterone, nucleoplasmin is hyper-phosphorylated, possibly by CK2. Nucleoplasmin then associates with the cytoplasmic mRNP particle in order to unmask these messages for translation (Meric *et al.*, 1997). Nucleoplasmin has been shown to partially relieve translational repression of H4 mRNA caused by the FGRY2 tail domain *in vitro* (Meric *et al.*, 1997). The tail domain of FRGY2 contains many basic/aromatic islands, which are thought to facilitate protein:protein interactions via electrostatic interactions between charged domains (Tafuri and Wolffe, 1992). On the other hand, nucleoplasmin is a very acidic protein and so could spontaneously bind to the basic islands of FRGY2, lending support to this interaction theory. Although the CSD is thought to be the main mRNA-binding domain of the Y-box proteins, it has been suggested that the CSD and tail

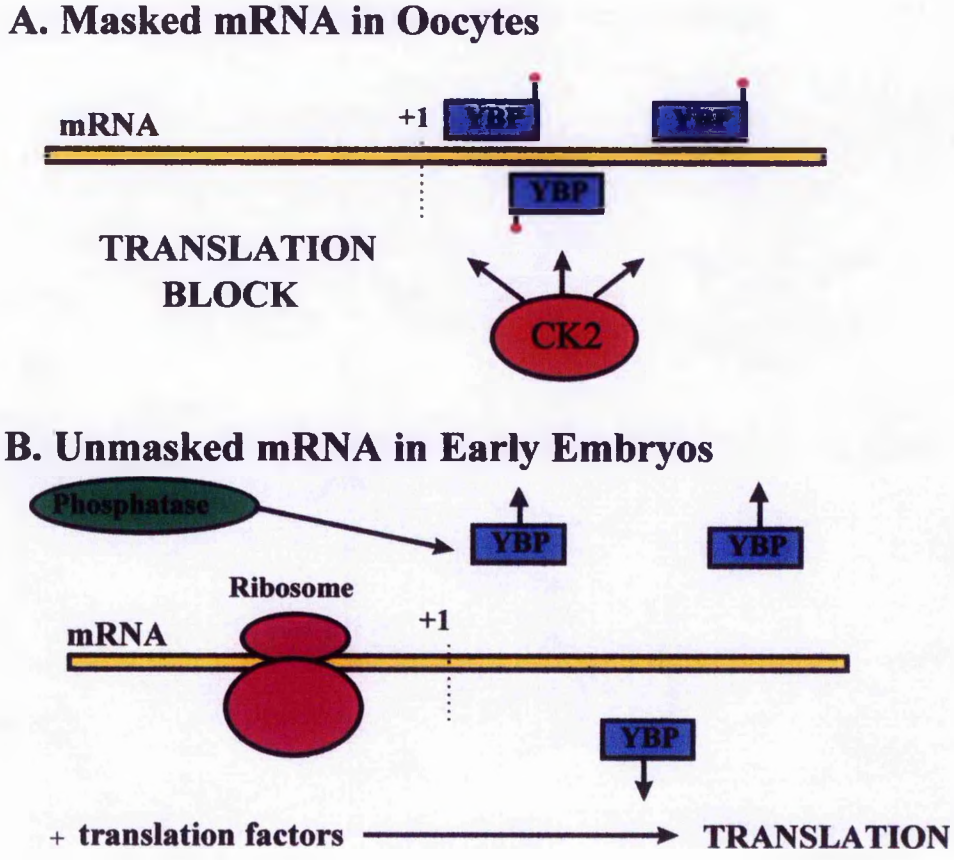



**Figure 55: Binding of Y-box proteins to mRNA mediated by CK2**: A) binding of Y-box proteins to mRNA is facilitated by CK2 phosphorylation (red dots) in the nucleus. B) Translationally inactive mRNP particles are unmasked by phosphatases during early embryogenesis.

domain bind cooperativity (Ladomery and Sommerville, 1994; Horwitz *et al.*, 1994). Thus the binding of nucleoplasmin to the tail domain of the Y-box proteins could directly affect the masking capabilities of the Y-box proteins. It must be said that this mechanism seems to lack specificity, since the nucleoplasmin is only one of many acidic proteins and already has a well-defined role of chaperoning H3/H4, a role which is continuing during the unmasking process.

Another identified mechanism for unmasking is the extension of the mRNA polyadenylation signal for some masked messages. The extension of the poly(A) tail that occurs at maturation is thought to affect the translational ability of mRNPs (Standart, 1992). It is unclear how the extension of the poly(A) should facilitate unmasking. Binding experiments have shown that the Y-box proteins are unable to bind poly(A) sites (Ladomery and Sommerville, 1994), so the interactions between these two seemingly unrelated events is unknown. The unmasking process is probably associated with mobilization of mRNPs by a polyadenylation-dependent mechanism which would involve the binding of unmasking proteins, which could be regulated by CK2 phosphorylation. The exact molecular mechanism is still yet undescribed.

### 6.3 Conclusion

It is clear that protein kinase CK2 is an important enzyme for oocyte development, whose diverse functions make it an integral part of the regulatory mechanisms which controls *Xenopus* oogenesis. It has important functions in both the nucleus and cytoplasm and may even act as part of the compartmentation mechanisms that exist between them. The control of activity of nuclear enzymes,

such as maternally expressed histone deacetylase, suggests an involvement of CK2 in the mechanism of chromosomal condensation and regulated gene inactivation. The association of CK2 with mRNPs, both in the nucleus and in the cytoplasm suggests that there might also be a regulatory role in translational masking and unmasking of the stored messages vital for oocyte maturation and early embryogenesis. The role of CK2 in regulating nuclear translocation, via the NLS, is only beginning to be understood. The compartmentalisation of proteins is one of the fundamental aspects that separate the eukaryotes from prokaryotes. The formation of an independent nucleus has provided the evolutionary stepping stone for the development of all higher organisms. Nuclear-cytoplasmic exchange is a fundamental process which must be fully explored and understood and its potential applications in fields such as gene therapy. For example, complexes containing nuclear localisation signals could aid in the delivering of replacement DNA or with the targeting of specific toxins to cancerous cells. Techniques that allow the block of nuclear transport of signalling molecules could be used to control gene expression and cell proliferation. Information obtained on protein transport into the nucleus could aid in the development of antiviral treatments.

### 6.4 Future Work

Much more research must be done (and is being done) in order to fully understand the function of CK2 in oocytes development. More work is needed to understand the interaction between α-importin and the NLS of HDACm, and how this might be mediated through phosphorylation by CK2. Other research, currently underway in this lab, is the attempted purification of enzymatically

active native HDACm in order to study its native phosphorylation state *in vivo* and to determine which phosphorylation sites are required for activity.

Attempts are being made to mutate individual CK2 phosphorylation sites within the GST-HDΔV clone. This would allow injection of the fusion protein, missing specific phosphorylated sites, in order to determine which sites are required for nuclear uptake. The new clones could also be used to study the effects on binding of α-importin with the NLS. Attempts should be made to mutate the surrounding acidic residues to block the binding site of CK2, thus keeping the phosphorylation sites intact while still preventing their phosphorylation. This would show which is important for nuclear import: the over all level of phosphorylation, or phosphorylation of specific serine/threonine amino acid residues within the transported protein. It has been found in experiments with large T-antigen that substitution of aspartic acids for phosphoserine/threonine sites results in the same increase in nuclear localisation as with the phosphorylated residues. It is thought that the overall negative charge, normally provided by phosphorylation, is what is important in regulating nuclear import (Jans and Jans, 1994). This indicates that the sequences themselves may be relatively unimportant, and that the phosphorylation state is simply affecting the ability of a number of possible sequence to bind the nuclear import proteins.

The purification of large quantities of native HDACm by immunoprecipitation, should be attempted in order to identify the phosphorylation sites that are used *in vivo*. It is important to determine the phosphorylation state of native proteins, because identification of these sites can

only be tentatively assigned by looking at the phosphorylation state of fusion proteins.

The identification of phosphorylated sites in the Y-box proteins must be completed, and the answers obtained will undoubtedly lead to more questions. What are the functions of the individual sites? Do they influence binding to mRNA and how? What other factors controls the phosphorylation of the Y-box proteins? Are some sites used by nuclear CK2 and are some used by cytoplasmic CK2? Why must CK2 be associated with the mRNP particle itself? Other experiments should be planned to investigate further the function of the Y-box proteins and the control of their binding activity to mRNAs. Attempts to mutate the specific phosphorylation sites within Y-box the proteins, FRGY2a and FRGY2b, should be undertaken, in order to show which sites are required for *in vivo* nuclear binding. The mechanism of unmasking of maternal mRNP should be studied to identify the role of the CK2 still associated with the cytoplasmic mRNP particles just prior to their translation. Interactions with other protein bound to the mRNP particles, such as the RNA helicase p54 (Ladomery *et al.*, 1997), should be studied to see if phosphorylation at specific stages of development influences the unwinding activity of this mRNP-associated protein.

CK2 has proven to be a multifunctional ubiquitous enzyme. Its potential in the regulation of cellular mechanisms cannot be denied. Many man hours have been devoted to the study of this enzyme and some questions have been answered, but undoubtedly many more questions have been raised.

# Appendices

## Appendix A: Protein Kinase CK2 α Data Base Entry

SWISS-PROT: P28020

ID KC22_XENLA STANDARD; PRT; 350 AA.
AC P28020;
DT 01-AUG-1992 (REL. 23, CREATED)
DT 01-AUG-1992 (REL. 23, LAST SEQUENCE UPDATE)
DT 01-NOV-1995 (REL. 32, LAST ANNOTATION UPDATE)
DE CASEIN KINASE II, ALPHA' CHAIN (CK II) (EC 2.7.1.37).
OS XENOPUS LAEVIS (AFRICAN CLAWED FROG).
OC EUKARYOTA; METAZOA; CHORDATA; VERTEBRATA; TETRAPODA; AMPHIBIA; ANURA.
RN [1]
RP SEQUENCE FROM N.A.
RC TISSUE=OVARY;
RX MEDLINE; 92183811. [NCBI, Geneva, Japan]
RA JEDLICKI A., HINRICHS M.V., ALLENDE C., ALLENDE J.E.;
RL FEBS LETT. 297:280-284(1992).
CC -!- FUNCTION: CASEIN KINASES ARE OPERATIONALLY DEFINED BY THEIR
CC PREFERENTIAL UTILIZATION OF ACIDIC PROTEINS SUCH AS CASEINS
CC AS SUBSTRATES.
CC -!- FUNCTION: THE ALPHA AND ALPHA' CHAINS CONTAIN THE CATALYTIC SITE.
CC -!- SUBUNIT: TETRAMER COMPOSED OF AN ALPHA CHAIN, AN ALPHA' AND TWO
CC BETA CHAINS.
DR EMBL; X62375; G64628; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
DR PIR; S18897; S18897.
DR PIR; S20404; S20404.
DR PROSITE; PS00107; PROTEIN_KINASE_ATP; 1.
DR PROSITE; PS00108; PROTEIN_KINASE_ST; 1.
DR PROSITE; PS50011; PROTEIN_KINASE_DOM; 1.
DR PRODOM [Domain structure / List of seq. sharing at least 1 domain]
DR PROTOMAP; P28020.
DR SWISS-2DPAGE; GET REGION ON 2D PAGE.
KW TRANSFERASE; SERINE/THREONINE-PROTEIN KINASE; ATP-BINDING.
FT DOMAIN 39 324 PROTEIN KINASE.
FT NP_BIND 45 53 ATP (BY SIMILARITY).
FT BINDING 68 68 ATP (BY SIMILARITY).
FT ACT_SITE 156 156 BY SIMILARITY.
SQ SEQUENCE 350 AA; 41454 MW; 8956ED02 CRC32;
MSGPVPSRAR VYTDVNTHRP RDYWDYESHV VEWGNQDDYQ LVRKLGRGKY SEVFEAINIT
NNEKVVVKIL KPVKKKKIKR EIKILENLRG GPNIITLADI VKDPVSRTPA LVFEHVNNTD
FKQLYQTLTD YDIRFYMYEI LKALDYCHSM GIMHRDVKPH NVMIDHEHRK LRLIDWGLAE
FYHPGQEYNV RVASRYFKGP ELLVDYQMYD YSLDMWSLGC MLASMIFRKE PFFHGHDNYD
QLVRIAKVLG TEDLYDYIDK YNIELDPRFN DILGRHSRKR WERFVHSENQ HLVSPEALDF
LDKLLRYDHQ TRLTAREAMD HPYFYPIVKD QSRMAALICP VAAHPSVAPV

ProtParam tool

KC22_XENLA (P28020)

DE CASEIN KINASE II, ALPHA' CHAIN (CK II) (EC 2.7.1.37).
OS XENOPUS LAEVIS (AFRICAN CLAWED FROG).

The computation has been carried out on the complete sequence.

Number of amino acids: 350

Molecular weight: 41454.4

Theoretical pI: 7.30

Amino acid composition:

| | | |
|---|---|---|
| Ala (A) | 17 | 4.9% |
| Arg (R) | 26 | 7.4% |
| Asn (N) | 15 | 4.3% |
| Asp (D) | 29 | 8.3% |
| Cys (C) | 3 | 0.9% |
| Gln (Q) | 10 | 2.9% |
| Glu (E) | 20 | 5.7% |
| Gly (G) | 14 | 4.0% |
| His (H) | 17 | 4.9% |
| Ile (I) | 21 | 6.0% |
| Leu (L) | 31 | 8.9% |
| Lys (K) | 23 | 6.6% |
| Met (M) | 11 | 3.1% |
| Phe (F) | 13 | 3.7% |
| Pro (P) | 18 | 5.1% |
| Ser (S) | 15 | 4.3% |
| Thr (T) | 11 | 3.1% |
| Trp (W) | 5 | 1.4% |
| Tyr (Y) | 23 | 6.6% |
| Val (V) | 28 | 8.0% |

| | | |
|---|---|---|
| Asx (B) | 0 | 0.0% |
| Glx (Z) | 0 | 0.0% |
| Xaa (X) | 0 | 0.0% |

Total number of negatively charged residues (Asp + Glu): 49
Total number of positively charged residues (Arg + Lys): 49

Extinction coefficients:

Conditions: 6.0 M guanidium hydrochloride

0.02 M phosphate buffer
pH 6.5

Extinction coefficients are in units of $M^{-1}$ $cm^{-1}$.

The first table lists values computed assuming ALL Cys residues appear as half cystines, whereas the second table assumes that NONE do.

| | 276 nm | 278 nm | 279 nm | 280 nm | 282 nm |
|---|---|---|---|---|---|
| Ext. coefficient | 60785 | 60581 | 59595 | 58250 | 55960 |
| Abs 0.1% (=1 g/l) | 1.466 | 1.461 | 1.438 | 1.405 | 1.350 |

| | 276 nm | 278 nm | 279 nm | 280 nm | 282 nm |
|---|---|---|---|---|---|
| Ext. coefficient | 60350 | 60200 | 59235 | 57890 | 55600 |
| Abs 0.1% (=1 g/l) | 1.456 | 1.452 | 1.429 | 1.396 | 1.341 |

Estimated half-life:

The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hour (mammalian reticulocytes, in vitro).
>20 hour (yeast, in vivo).
>10 hour (Escherichia coli, in vivo).

Instability index:

The instability index (II) is computed to be 44.10
This classifies the protein as unstable.
Aliphatic index: 86.00

Grand average of hydropathicity (GRAVY): -0.524

## Appendix B: Protein Kinase CK2 β Data Base Entry

SWISS-PROT: P28021

ID KC2B_XENLA STANDARD; PRT; 215 AA.
AC P28021;
DT 01-AUG-1992 (REL. 23, CREATED)
DT 01-AUG-1992 (REL. 23, LAST SEQUENCE UPDATE)
DT 01-NOV-1995 (REL. 32, LAST ANNOTATION UPDATE)
DE CASEIN KINASE II, BETA CHAIN (CK II) (EC 2.7.1.37) (PHOSVITIN).
OS XENOPUS LAEVIS (AFRICAN CLAWED FROG).
OC EUKARYOTA; METAZOA; CHORDATA; VERTEBRATA; TETRAPODA; AMPHIBIA; ANURA.
RN [1]
RP SEQUENCE FROM N.A.
RC TISSUE=OVARY;
RX MEDLINE; 92183811. [NCBI, Geneva, Japan]
RA JEDLICKI A., HINRICHS M.V., ALLENDE C., ALLENDE J.E.;
RL FEBS LETT. 297:280-284(1992).
CC -!- FUNCTION: CASEIN KINASES ARE OPERATIONALLY DEFINED BY THEIR
CC PREFERENTIAL UTILIZATION OF ACIDIC PROTEINS SUCH AS CASEINS
CC AS SUBSTRATES. THE EXACT FUNCTION OF THE BETA CHAIN IS NOT KNOWN
CC (BY SIMILARITY).
CC -!- SUBUNIT: TETRAMER COMPOSED OF AN ALPHA CHAIN, AN ALPHA' AND TWO
CC BETA CHAINS.
CC -!- PTM: PHOSPHORYLATED BY ALPHA CHAIN (BY SIMILARITY).
DR EMBL; X62376; G64630; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
DR PIR; S18898; S18898.
DR PIR; S20405; S20405.
DR PROSITE; PS01101; CK2_BETA; 1.
DR PRODOM [Domain structure / List of seq. sharing at least 1 domain]
DR PROTOMAP; P28021.
DR SWISS-2DPAGE; GET REGION ON 2D PAGE.
KW TRANSFERASE; SERINE/THREONINE-PROTEIN KINASE; PHOSPHORYLATION.
FT MOD_RES 2 2 PHOSPHORYLATION (AUTO-) (PROBABLE).
FT DOMAIN 55 64 ASP/GLU-RICH (ACIDIC).
SQ SEQUENCE 215 AA; 24960 MW; 010460F4 CRC32;
MSSSEEVSWI SWFCGLRGNE FFCEVDEDYI QDKFNLTGLN EQVPHYRQAL DMILDLEPDE
ELEDNPNQSD LIEQAAEMLY GLIHARYILT NRGIAQMLEK YQQGDFGYCP RVYCENQPML
PIGLSDIPGE AMVKLYCPKC MDVYTPKSSR HHHTDGAYFG TGFPHMLFMV HPEYRPKRPA
NQFVPRLYGF KIHPMAYQLQ LQAASNFKSP VKTMR

ProtParam tool

KC2B_XENLA (P28021)

DE CASEIN KINASE II, BETA CHAIN (CK II) (EC 2.7.1.37) (PHOSVITIN).
OS XENOPUS LAEVIS (AFRICAN CLAWED FROG).

The computation has been carried out on the complete sequence.

Number of amino acids: 215

Molecular weight: 24960.4

Theoretical pI: 5.33

Amino acid composition:

| | | |
|---|---|---|
| Ala (A) | 11 | 5.1% |
| Arg (R) | 10 | 4.7% |
| Asn (N) | 9 | 4.2% |
| Asp (D) | 12 | 5.6% |
| Cys (C) | 6 | 2.8% |
| Gln (Q) | 13 | 6.0% |
| Glu (E) | 16 | 7.4% |
| Gly (G) | 13 | 6.0% |
| His (H) | 8 | 3.7% |
| Ile (I) | 10 | 4.7% |
| Leu (L) | 19 | 8.8% |
| Lys (K) | 9 | 4.2% |
| Met (M) | 11 | 5.1% |
| Phe (F) | 11 | 5.1% |
| Pro (P) | 16 | 7.4% |
| Ser (S) | 11 | 5.1% |
| Thr (T) | 6 | 2.8% |
| Trp (W) | 2 | 0.9% |
| Tyr (Y) | 13 | 6.0% |
| Val (V) | 9 | 4.2% |
| | | |
| Asx (B) | 0 | 0.0% |
| Glx (Z) | 0 | 0.0% |
| Xaa (X) | 0 | 0.0% |

Total number of negatively charged residues (Asp + Glu): 28
Total number of positively charged residues (Arg + Lys): 19

Extinction coefficients:

Conditions: 6.0 M guanidium hydrochloride
0.02 M phosphate buffer
pH 6.5

Extinction coefficients are in units of $M^{-1}$ $cm^{-1}$.

The first table lists values computed assuming ALL Cys residues appear as half cystines, whereas the second table assumes that NONE do.

| | 276 nm | 278 nm | 279 nm | 280 nm | 282 nm |
|---|---|---|---|---|---|
| Ext. coefficient | 30520 | 30162 | 29525 | 28740 | 27520 |
| Abs 0.1% (=1 g/l) | 1.223 | 1.208 | 1.183 | 1.151 | 1.103 |

| | 276 nm | 278 nm | 279 nm | 280 nm | 282 nm |
|---|---|---|---|---|---|
| Ext. coefficient | 29650 | 29400 | 28805 | 28020 | 26800 |
| Abs 0.1% (=1 g/l) | 1.188 | 1.178 | 1.154 | 1.123 | 1.074 |

Estimated half-life:

The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hour (mammalian reticulocytes, in vitro).
>20 hour (yeast, in vivo).
>10 hour (Escherichia coli, in vivo).

Instability index:

The instability index (II) is computed to be 39.13
This classifies the protein as stable.

Aliphatic index: 69.86

Grand average of hydropathicity (GRAVY): -0.473

## Appendix C: Histone Deacetylase Data Base Entry

SWISS-PROT: Q91695

ID HD11_XENLA STANDARD; PRT; 480 AA.
AC Q91695;
DT 01-FEB-1998 (REL. 36, CREATED)
DT 01-FEB-1998 (REL. 36, LAST SEQUENCE UPDATE)
DT 01-FEB-1998 (REL. 36, LAST ANNOTATION UPDATE)
DE PROBABLE HISTONE DEACETYLASE 1-1 (HD1) (MATERNALLY-EXPRESSED
DE HISTONE DEACETYLASE) (HDM) (AB21).
OS XENOPUS LAEVIS (AFRICAN CLAWED FROG).
OC EUKARYOTA; METAZOA; CHORDATA; VERTEBRATA; TETRAPODA; AMPHIBIA;
ANURA.
RN [1]
RP SEQUENCE FROM N.A.
RC TISSUE=OOCYTE;
RX MEDLINE; 98036059. [NCBI, Geneva, Japan]
RA LADOMERY M.R., LYONS S., SOMMERVILLE J.;
RL GENE 198:275-280(1997).
CC -!- FUNCTION: RESPONSIBLE FOR THE DEACETYLATION OF LYSINE RESIDUES
ON
CC THE N-TERMINAL PART OF THE CORE HISTONES (H2A, H2B, H3 AND H4).
CC HISTONE DEACETYLATION PLAYS AN IMPORTANT ROLE IN
TRANSCRIPTIONAL
CC REGULATION, CELL CYCLE PROGRESSION AND DEVELOPMENTAL EVENTS
(BY
CC SIMILARITY).
CC -!- SUBCELLULAR LOCATION: NUCLEAR (BY SIMILARITY).
CC -!- TISSUE SPECIFICITY: OOCYTE.
CC -!- DEVELOPMENTAL STAGE: ACCUMULATES IN PREVITELLOGENIC OOCYTES
AND IS
CC MAINTAINED AT CONSTANT LEVEL THROUGHOUT OOGENESIS AND INTO
EARLY
CC EMBRYOGENESIS. DECLINES THROUGH GASTRULA TO NEURULA. NOT
CC DETECTABLE BETWEEN NEURULA AND TAILBUD, NOR IN ADULT TISSUES
OTHER
CC THAN OVARY.
CC -!- SIMILARITY: BELONGS TO THE HISTONE DEACETYLASE / ACUC / APHA
CC FAMILY.
DR EMBL; X78454; G602098; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
DR PRODOM [Domain structure / List of seq. sharing at least 1 domain]
DR PROTOMAP; Q91695.
DR SWISS-2DPAGE; GET REGION ON 2D PAGE.
KW HYDROLASE; NUCLEAR PROTEIN.
FT DOMAIN 299 302 POLY-GLY.
SQ SEQUENCE 480 AA; 54747 MW; 014FAEB5 CRC32;
MALTLGTKKK VCYYYDGDVG NYYYGQGHPM KPHRIRMTHN LLLNYGLYRK MEIFRPHKAS
AEDMTKYHSD DYIKFLRSIR PDNMSEYSKQ MQRFNVGEDC PVFDGLFEFC QLSAGGSVAS

AVKLNKQQTD ISVNWSGGLH HAKKSEASGF CYVNDIVLAI LELLKYHQRV VYIDIDIHHG
DGVEEAFYTT DRVMTVSFHK YGEYFPGTGD LRDIGAGKGK YYAVNYALRD GIDDESYEAI
FKPVMSKVME MFQPSAVVLQ CGADSLSGDR LGCFNLTIKG HAKCVEFIKT FNLPLLMLGG
GGYTIRNVAR CWTYETAVAL DSEIPNELPY NDYFEYFGPD FKLHISPSNM TNQNTNEYLE
KIKQRLFENL RMLPHAPGVQ MQAVAEDSIH DDSGEEDEDD PDKRISIRSS DKRIACDEEF
SDSEDEGEGG RKNVANFKKV KRVKTEEEKE GEDKKDVKEE EKAKDEKTDS KRVKEETKSV

ProtParam tool

HD11_XENLA (Q91695)

DE PROBABLE HISTONE DEACETYLASE 1-1 (HD1) (MATERNALLY-EXPRESSED
DE HISTONE DEACETYLASE) (HDM) (AB21).
OS XENOPUS LAEVIS (AFRICAN CLAWED FROG).

The computation has been carried out on the complete sequence.

Number of amino acids: 480

Molecular weight: 54747.5

Theoretical pI: 5.50

Amino acid composition:

Ala (A) 26 5.4%
Arg (R) 22 4.6%
Asn (N) 21 4.4%
Asp (D) 39 8.1%
Cys (C) 9 1.9%
Gln (Q) 13 2.7%
Glu (E) 41 8.5%
Gly (G) 37 7.7%
His (H) 15 3.1%
Ile (I) 23 4.8%
Leu (L) 32 6.7%
Lys (K) 42 8.8%
Met (M) 15 3.1%
Phe (F) 21 4.4%
Pro (P) 16 3.3%
Ser (S) 30 6.2%
Thr (T) 19 4.0%
Trp (W) 2 0.4%
Tyr (Y) 27 5.6%
Val (V) 30 6.2%
Asx (B) 0 0.0%
Glx (Z) 0 0.0%
Xaa (X) 0 0.0%

Total number of negatively charged residues (Asp + Glu): 80
Total number of positively charged residues (Arg + Lys): 64

Extinction coefficients:

Conditions: 6.0 M guanidium hydrochloride
0.02 M phosphate buffer
pH 6.5

Extinction coefficients are in units of $M^{-1}$ $cm^{-1}$.

The first table lists values computed assuming ALL Cys residues appear as half cystines, whereas the second table assumes that NONE do.

| | 276 nm | 278 nm | 279 nm | 280 nm | 282 nm |
|---|---|---|---|---|---|
| Ext. coefficient | 51255 | 50143 | 48715 | 47020 | 44680 |
| Abs 0.1% (=1 g/l) | 0.936 | 0.916 | 0.890 | 0.859 | 0.816 |

| | 276 nm | 278 nm | 279 nm | 280 nm | 282 nm |
|---|---|---|---|---|---|
| Ext. coefficient | 49950 | 49000 | 47635 | 45940 | 43600 |
| Abs 0.1% (=1 g/l) | 0.912 | 0.895 | 0.870 | 0.839 | 0.796 |

Estimated half-life:

The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hour (mammalian reticulocytes, in vitro).
>20 hour (yeast, in vivo).
>10 hour (Escherichia coli, in vivo).

Instability index:
The instability index (II) is computed to be 40.12
This classifies the protein as unstable.

Aliphatic index: 68.23

Grand average of hydropathicity (GRAVY): -0.660

## Appendix D: FGRY2 Data Base Entry

SWISS-PROT: P21574

```
ID  YB56_XENLA    STANDARD;      PRT;  336 AA.
AC  P21574;
DT  01-MAY-1991 (REL. 18, CREATED)
DT  01-NOV-1995 (REL. 32, LAST SEQUENCE UPDATE)
DT  01-NOV-1995 (REL. 32, LAST ANNOTATION UPDATE)
DE  CYTOPLASMIC RNA-BINDING PROTEIN P56 (Y BOX BINDING PROTEIN-2) (Y-
BOX
DE  TRANSCRIPTION FACTOR) (MRNP4).
GN  FRGY2.
OS  XENOPUS LAEVIS (AFRICAN CLAWED FROG).
OC  EUKARYOTA; METAZOA; CHORDATA; VERTEBRATA; TETRAPODA; AMPHIBIA;
ANURA.
RN  [1]
RP  SEQUENCE FROM N.A.
RX  MEDLINE; 91062413. [NCBI, Geneva, Japan]
RA  TAFURI S.R., WOLFFE A.P.;
RL  PROC. NATL. ACAD. SCI. U.S.A. 87:9028-9032(1990).
RN  [2]
RP  SEQUENCE FROM N.A., AND PARTIAL SEQUENCE.
RX  MEDLINE; 92107999. [NCBI, Geneva, Japan]
RA  MURRAY M.T., SCHILLER D.L., FRANKE W.W.;
RL  PROC. NATL. ACAD. SCI. U.S.A. 89:11-15(1992).
RN  [3]
RP  PARTIAL SEQUENCE.
RC  TISSUE=OVARY;
RX  MEDLINE; 92332467. [NCBI, Geneva, Japan]
RA  DESCHAMPS S., VIEL A., GARRIGOS M., DENIS H., LE MAIRE M.;
RL  J. BIOL. CHEM. 267:13799-13802(1992).
RN  [4]
RP  PARTIAL SEQUENCE.
RC  TISSUE=OVARY;
RX  MEDLINE; 91224309. [NCBI, Geneva, Japan]
RA  DESCHAMPS S., VIEL A., DENIS H., LE MAIRE M.;
RL  FEBS LETT. 282:110-114(1991).
CC  -!- FUNCTION: BINDS TO CCAAT-CONTAINING Y BOX OF THE HSP70 GENES.
CC      SEEMS TO BE A NEGATIVE REGULATORY FACTOR. ALSO BINDS TO MRNA.
CC  -!- SUBUNIT: POSSIBLY FORMS A HETERODIMER WITH P54 IN THE 6S AND 15S
CC      MRNA-BINDING PARTICLES.
CC  -!- SUBCELLULAR LOCATION: CYTOPLASMIC, EITHER FREE OR ASSOCIATED
WITH
CC      RIBONUCLEOPROTEIN PARTICLES.
CC  -!- TISSUE SPECIFICITY: TESTIS AND IMMATURE OOCYTES.
CC  -!- PTM: PHOSPHORYLATION ACTIVATES IN VITRO RNA-BINDING.
CC  -!- SIMILARITY: BELONGS TO THE COLD-SHOCK DOMAIN (CSD) FAMILY.
DR  EMBL; M59454; G214157; -. [EMBL / GenBank / DDBJ] [CoDingSequence]
DR  PIR; B38274; B38274.
```

DR HSSP; P15277; 1MJC. [HSSP ENTRY / SWISS-3DIMAGE /
DR PDB-ENTRY / PDB-RASMOL / PDB-3DIMAGE]
DR PROSITE; PS00352; COLD_SHOCK; 1.
DR PRODOM [Domain structure / List of seq. sharing at least 1 domain]
DR PROTOMAP; P21574.
DR SWISS-2DPAGE; GET REGION ON 2D PAGE.
KW TRANSCRIPTION REGULATION; DNA-BINDING; NUCLEAR PROTEIN;
KW RNA-BINDING; PHOSPHORYLATION.
FT DOMAIN 44 108 CSD.
FT CONFLICT 254 254 A -> T (IN REF. 1).
SQ SEQUENCE 336 AA; 37202 MW; ECB4DC87 CRC32;
MSEAEAQEPE PVPQPESEPE IQKPGIAAAR NQANKKVLAT QVQGTVKWFN VRNGYGFINR
NDTKEDVFVH QTAIKKNNPR KFLRSVGDGE TVEFDVVEGE KGAEAANVTG PGGVPVKGSR
FAPNRRRFRR RFYRPRADTA GESGGEGVSP EQMSEGERGE ETSPQQRPQR RRPPPFFYRR
RFRRGPRPNN QQNQGAEVTE QSENKDPVAP TSEALASGDD PQRPPPRRFR QRFRRPFRPR
PAPQQTPEGG DGEAKAESGE DPRPEPQRQR NRPYVQRRRR QGATQVAATA QGEGKAEPTQ
HPASEEGTPS DSPTDDGAPV QSSAPDPGIA DTPAPE

ProtParam tool

YB56_XENLA (P21574)

DE CYTOPLASMIC RNA-BINDING PROTEIN P56 (Y BOX BINDING PROTEIN-2) (Y-BOX
DE TRANSCRIPTION FACTOR) (MRNP4).
OS XENOPUS LAEVIS (AFRICAN CLAWED FROG).

The computation has been carried out on the complete sequence.

Number of amino acids: 336

Molecular weight: 37202.6

Theoretical pI: 9.60

Amino acid composition:

Ala (A) 31 9.2%
Arg (R) 42 12.5%
Asn (N) 15 4.5%
Asp (D) 15 4.5%
Cys (C) 0 0.0%
Gln (Q) 27 8.0%
Glu (E) 34 10.1%
Gly (G) 31 9.2%
His (H) 2 0.6%
Ile (I) 5 1.5%
Leu (L) 3 0.9%
Lys (K) 13 3.9%

Met (M) 2 0.6%
Phe (F) 14 4.2%
Pro (P) 43 12.8%
Ser (S) 17 5.1%
Thr (T) 17 5.1%
Trp (W) 1 0.3%
Tyr (Y) 4 1.2%
Val (V) 20 6.0%
Asx (B) 0 0.0%
Glx (Z) 0 0.0%
Xaa (X) 0 0.0%

Total number of negatively charged residues (Asp + Glu): 49
Total number of positively charged residues (Arg + Lys): 55

Extinction coefficients:

Conditions: 6.0 M guanidium hydrochloride
0.02 M phosphate buffer
pH 6.5

Extinction coefficients are in units of $M^{-1}$ $cm^{-1}$.

| | 276 nm | 278 nm | 279 nm | 280 nm | 282 nm |
|---|---|---|---|---|---|
| Ext. coefficient | 11200 | 11200 | 11040 | 10810 | 10400 |
| Abs 0.1% (=1 g/l) | 0.301 | 0.301 | 0.297 | 0.291 | 0.280 |

Estimated half-life:

The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hour (mammalian reticulocytes, in vitro).
>20 hour (yeast, in vivo).
>10 hour (Escherichia coli, in vivo).

Instability index:

The instability index (II) is computed to be 81.71
This classifies the protein as unstable.

Aliphatic index: 35.77

Grand average of hydropathicity (GRAVY): -1.371

# Appendix E: CSD Alignment

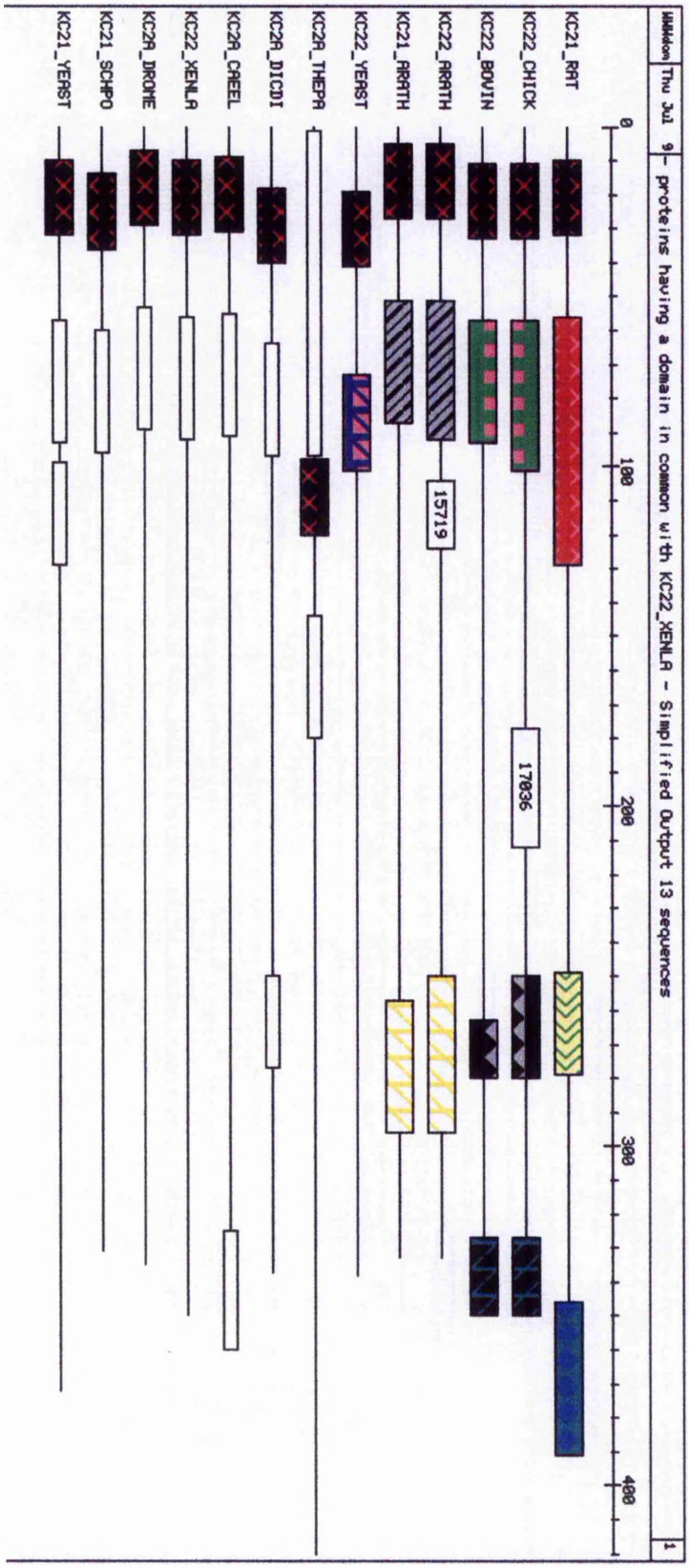
Domain 597

(Prodom Release 34.2)

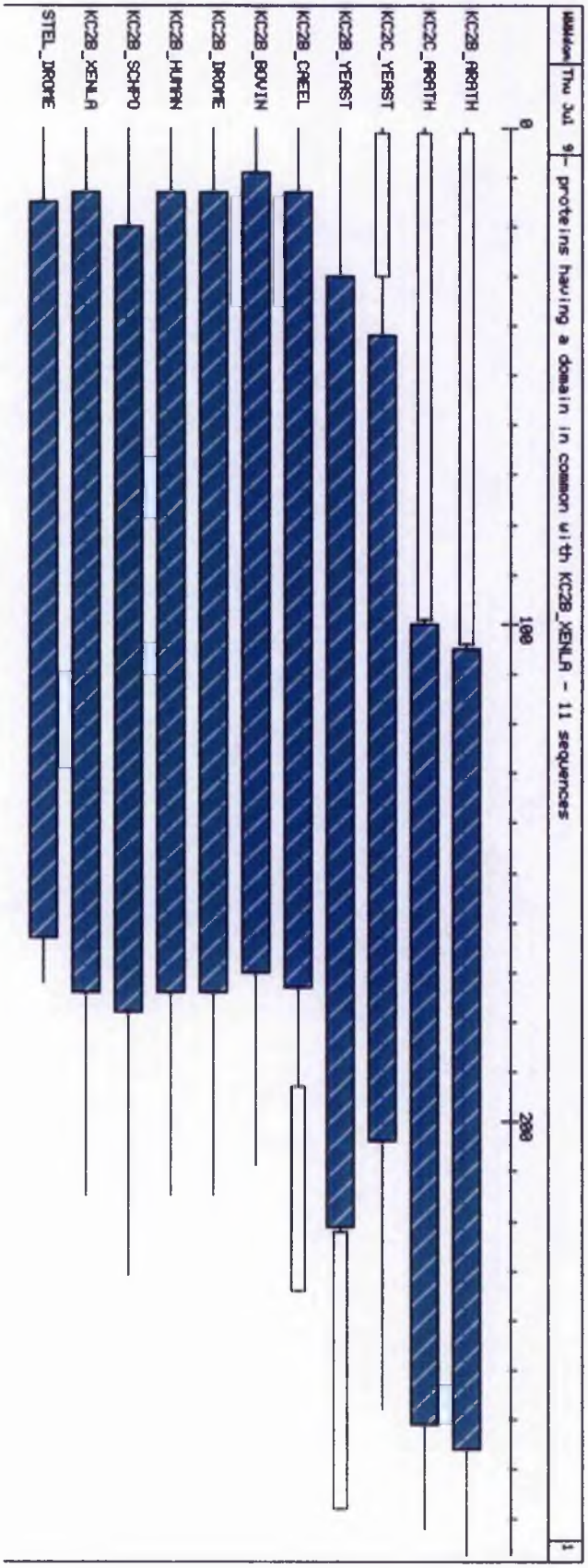
Domain ID: 597/ (ProDom34.2)
Number of sequences in family: 50
Most frequent protein names: CSPB(8) CSPA(6) CSPD(4)
Commentary (automatic): PROTEIN COLD SHOCK SHOCK-LIKE FACTOR A TRANSCRIPTION
CSPB Y-BOX I

```
                                    wt  --------10--------20--------30--------40--------50--------60
CAPA_PSEFR          7     59      1.14 GTVKWFNDEKGFGFITPQGGGDDLFVHFKAIESDG----FKSLKEGQTVSFVAEKGQ
CAPB_PSEFR          7     58      1.14 GTVKWFNDEKGFGFITPQSG-DDLFVHFKAIQSDG----FKSLKEGQQVSFIATRGQ
TAPB_PSEFR          7     58      1.14 GTVKWFNDEKGFGFITPESG-PDLFVHFRAIQGNG----FKSLKEGQKVTFIAVQGQ
CSPA_PSEAE          7     58      1.14 GTVKWFNDAKGFGFITPESG-NDLFVHFRSIQGTG----FKSLQEGQKVSFVVVNGQ
TAPA_PSEFR          1     47      1.50 .....FNDEKGFGFITPESG-PDLFVHFRAIQGNG----FKSLKEGQKVTFIAVQGQ
CSPB_BACGL          1     50      1.02 ..VKWFNSEKGFGLIEVEGQ-DDVFVHFSAIQGEG----FKTLEESQAVSFEIVEGN
CSPB_BACGO          1     50      0.93 ..IKWFNSEKGFGFIEVEGQ-DDVFVHFSAIQGEG----FKCLEEGQAVSFEIVEGN
CSPB_BACSU          4     55      0.93 GKVKWFNSEKGFGFIEVEGQ-DDVFVHFSAIQGEG----FKTLEEGQAVSFEIVEGN
CSPB_BACCE          3     54      1.02 GKVKWFNNEKGFGFIEMEGS-EDVFVHFSAIQSDG----YKALEEGQEVSFDITEGN
CSPD_BACCE          4     55      0.90 GKVKWFNGEKGFGFIEVEGG-EDVFVHFSAIQGDG----FKTLEEGQEVSFEIVDGN
CSPB_BACCL          4     55      0.72 GKVKWFNNEKGYGFIEVEGG-SDVFVHFTAIQGEG----FKTLEEGQEVSFEIVQGN
CSPB_BACST          4     55      0.72 GKVKWFNNEKGYGFIEVEGG-SDVFVHFTAIQGEG----FKSLEEGQEVSFEIVQGN
CSPD_BACSU          4     55      0.81 GKVKWFNNEKGFGFIEVEGG-DDVFVHFTAIEGDG----YKSLEEGQEVSFEIVEGN
CSPB_LISMO          4     54      1.14 GTVKWFNSEKGFGFIEVEGG-DDIFVHFSAIEGEG----FKTLDEGQSVEFEIVEG.
CSPA_BACCE          5     56      1.23 GQVKWFNNEKGFGFIEVPGE-NDVFVHFSAIETDG----FKSLEEGQKVSFEIEDGN
CSPA_LISMO          4     54      0.99 GTVKWFNAEKGFGFIERENG-DDVFVHFSAIQGDG----FKSLDEGQAVTFDVEEG.
CSPC_BACSU          4     55      0.99 GTVKWFNAEKGFGFIERENG-DDVFVHFSAIQSDG----FKSLDEGQKVSFDVEQGA
CSPC_BACCE          3     54      1.17 GRVKWFNAEKGFGFIEREDG-DDVFVHFSAIQQDG----YKSLEEGQQVEFDIVDGA
CSPE_BACCE          5     52      1.53 GKVKWFNSEKGFGFIEVADG-SDVFVHFSAITGDG----FKSLDEGQEVSFEV....
CSP1_LACPL          4     52      1.29 GTVKWFNADKGYGFITGEDG-NDVFVHFSAIQTDG----FKTLEEGQKVTFDEE...
CSP2_LACPL          4     54      1.29 GTVKWFNADKGFGFITGEDG-TDVFVHFSAIQTDG----FKTLDEGQKVTYDEEQG.
CSPA_STIAU          4     56      1.32 GTVKWFNDAKGFGFITQDGGGEDVFCHHSAINMDG----FRTLQEGQKVEFEVTRGP
CSPD_ECOLI          4     56      1.32 GTVKWFNNAKGFGFICPEGGGEDIFAHYSTIQMDG----YRTLKAGQSVQFDVHQGP
CSP7_STRCL          4     56      1.23 GTVKWFNAEKGFGFIAQDGGGPDVFVHYSAINATG----FRSLEENQVVNFDVTHGE
CSPF_STRCO          4     56      1.23 GTVKWFNSEKGFGFIAQDGGGPDVFAHYSNINAQG----YRELQEGQAVTFDITQGQ
CSPD_HAEIN          4     56      1.62 GIVKWFNNAKGFGFISAEGVDADIFAHYSVIEMDG----YRSLKAGQKVQFEVLHSD
CSPI_ECOLI          7     58      1.14 GLVKWFNADKGFGFITPDDGSKDVFVHFTAIQSNE----FRTLNENQKVEFSIEQG.
CSPJ_ECOLI          7     58      1.14 GLVKWFNPEKGFGFITPKDGSKDVFVHFSAIQSND----FKTLTENQEVEFGIENG.
CSPA_ECOLI          6     58      0.75 GIVKWFNADKGFGFITPDDGSKDVFVHFSAIQNDG----YKSLDEGQKVSFTIESGA
CSPA_SALTY          6     58      0.75 GIVKWFNADKGFGFITPDDGSKDVFVHFSAIQNDG----YKSLDEGQKVSFTIESGA
CSPB_ECOLI          7     59      1.05 GLVKWFNADKGFGFISPVDGSKDVFVHFSAIQNDN----YRTLFEGQKVTFSIESGA
CSPC_ECOLI          5     57      1.08 GQVKWFNESKGFGFITPADGSKDVFVHFSAIQGNG----FKTLAEGQNVEFEIQDGQ
CSPE_ECOLI          5     57      1.08 GNVKWFNESKGFGFITPEDGSKDVFVHFSAIQTNG----FKTLAEGQRVEFEITNGA
CSP_ARTGO           4     56      1.50 GTVKWFNAEKGFGFITPDDSDGDVFVHYSEIQTGG----FKTLDENARVQFEIGQGA
GRP2_NICSY         11     63      1.56 GTVKWFSDQKGFGFITPDDGGEDLFVHQSGIRSEG----FRSLAEGETVEFEVESGG
Y4CH_RHISN          5     53      2.06 GTVKWFNATKGFGFIQPDDGSADVFVHISAVERAG----LRELKDGQKISYEL....
CBFX_HUMAN         61    117      0.09 GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEFDVVEGE
CBFX_MOUSE         59    115      0.09 GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEFDVVEGE
CBFX_RAT           59    115      0.09 GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEFDVVEGE
DBPA_HUMAN         93    149      0.09 GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEFDVVEGE
YB1_CHICK          58    114      0.09 GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEFDVVEGE
YB3_XENLA          39     95      0.09 GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEFDVVEGE
YB1_XENLA          39     95      0.09 GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEFDVVEGE
YB1_MOUSE          59    115      0.09 GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEFDVVEGE
YB1_HUMAN          61    117      0.09 GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEFDVVEGE
YB56_XENLA         44    100      0.33 GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKFLRSVGDGETVEFDVVEGE
YB54_XENLA         44    100      0.42 GTVKWFNVRNGYGFINRNDSKEDVFVHQTAIKKNNPRKFLRSVGDGETVEFDVVEGE
YBFH_APLCA         35     91      1.08 GTVKWFNVKSGYGFINRDDTKEDVFVHQTAIVKNNPRKYLRSVGDGEKVEFDVVEGE
CSPF_ECOLI          7     59      2.87 GIVKTFDGKSGKGLITPSDGRIDVQLHVSALNLRD----AEEITTGLRVEFCRINGL
CSPG_SALTY          7     40      3.02 GLVKWFNPEKGFGFITPKDGSKDVFVHFSAIQSN.....................
consensus                              GTVKWFNSEKGFGFITPEDGSEDVFVHFSAIQSDG----FKSLEEGQKVSFEIVEGQ
```

## **Appendix F**: CK2α Shared Domains



## Appendix G: CK2β Shared Domains



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# Papers

# Nuclear uptake of histone deacetylase in *Xenopus* oocytes is regulated by phosphorylation

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Running title: **Phosphorylation of histone deacetylase**

**Much interest has focused recently on the regulatory role played by reversible acetylation of core histones during the transcription and replication of chromatin. The acetylation status of chromatin is determined by the equilibrium between the activities of histone acetyltransferases and histone deacetylases. The *Xenopus* protein HDm shows sequence homology to other putative histone deacetylases but is expressed only during early development, its proposed activity being to stabilize newly-replicated chromatin during blastula formation. Previous studies have shown that the 57 kDa HDm protein undergoes steady accumulation into the oocyte nucleus where it is organized in a multiprotein complex of approximately 360 kDa. This complex has histone deacetylase activity that is sensitive to trichostatin A and alkaline phosphatase treatment. Two regions of HDm are identified: the conserved N-terminal region which represent the enzyme core and a more variable, highly-charged C-terminal region, the regulatory features of which are examined here. The C-terminal region is required for nuclear uptake, regulation of which is promoted by phosphorylation at specific sites by the protein kinase CK2.**

Key words: histones/chromatin; protein phosphorylation; nuclear import

**Introduction**

Modification of core histones by acetylation has long been recognized as having important consequences for chromatin structure and how this relates to the processes of transcription and replication. The acetylation status of stored histones (Almouzni *et al.*, 1994), of histones being incorporated into newly-synthesized chromatin (Sobel *et al.*, 1995), of chromatin segments being activated for transcription () and of chromatin segments being silenced (), is determined by the relative activities of histone acetyltransferases (HATs) and histone deacetylases (HDs). Targets for these enzymes exist on all four core histones but histones H3 and H4 appear to be preferred substrates, the best characterized sites being the four residues, Lys5, Lys8, Lys12 and Lys16, located in the N-terminal region of H4 (Turner *et al.*, 1992). Recent cloning of HAT (Brownell *et al.*, 1996) and HD genes (Taunton *et al.*, 1996) and identification of their proteins have given renewed impetus to exploring the diverse molecular mechanisms involving reversible acetylation.

Early embryogenesis in *Xenopus* proceeds from a fertilized egg to a blastula of ~10,000 cells in a series of 13-14 rapid cell divisions. Throughout this period there is no transcriptional activity and the assembly of new chromatin from almost continuously replicating DNA is largely dependent on a maternal pool of histones and assembly factors. In general, chromatin assembly involves the association of pre-acetylated core histones with replicating DNA and subsequent stabilization of nucleosomes by histone deacetylation. Newly-synthesized histone H4 is acetylated at defined lysine residues by a cytoplasmic histone acetyltransferase (HAT B; Sobel *et al.*, 1994). Sequencing of the amino-terminus of newly-synthesized histone H4 from a range of different organisms has shown it to be diacetylated, at lysine residues 5 and 12 (Sobel *et al.*, 1995), although yeast HAT B activity on histone H4 *in vitro* shows some variation from this pattern (Kleff *et al.*, 1995; Parthun *et al.*, 1996). Deacetylation would normally occur soon after histone deposition in new chromatin but information is only now becoming available as to how histones are accessed by histone deacetylase (HD) and to how the deacetylation reaction is regulated.

The aspect of histone deacetylation that has received most attention recently is its role in the regulation, most often repression, of the activity of specific sets of genes (reviewed by Wolffe, 1996, 1997). For instance, association of HD1 with the adaptor protein RbAp48 (Taunton *et al.*, 1996), or with the corepressor of the mammalian Mad/Max complex, Sin3 (Alland *et al.*, 1997), or with N-CoR, the corepressor of the thyroid hormone receptor (Heinzel *et al.*, 1997), may serve to target the deacetylase complex to specific chromatin sites. Such interactions appear to be restricted to modulation of transcriptional activity of specific genes recognized though the presence of transcription factors, such as Mad-Max and the thyroid hormone receptor, localized to their promoters. The more extensive deacetylation reactions

occurring after replication may involve alternative components. Two, chromatographically-separable, multiprotein complexes containing histone deacetylases, designated HDA and HDB, have been described in yeast (Carmen *et al.*, 1996; Rundlett *et al.*, 1996) but, apart from the deacetylases themselves, other protein components have not been identified.

*Xenopus AB21* (data bank accession number: X78454) was initially recognized as a homologue of the yeast gene regulator *RPD3* (Vidal and Gabor, 1991) which was later shown also to be a homologue of human *HD1* (Taunton et al., 1996). There has now been cloned and identified an additional series of putative histone deacetylases - in yeast (Rundlett et al., 1996), *Caenorhabditis* (Waterston et al., 1992), *Drosophila* (De Rubertis *et al.*, 1996), and mouse () - all of which appear to have a conserved region stretching from near the amino terminus to more than half-way through the protein sequence. It is likely that this conserved region represents the enzyme core responsible for deacetylase activity (Ladomery et al., 1997).

The mRNA encoded by *Xenopus AB21* is expressed only in oocytes, remains stable in embryos up to neurula and has sequence characteristics of a maternal message. Its protein product HDm (maternal histone deacetylase; Ladomery et al., 1997) is synthesized during oogenesis, is stored through oocyte maturation,fertilization and early cleavage and decreases in amount after mid-blastula, when the cell-cycle time slows down to normal and cells start to differentiate. All of the HD activity detected in oocytes and early embryos can be accounted for by the presence of a 360 kDa protein complex that contains the HDm protein and this complex appears to fulfill the conditions required of a 'deposition' histone deacetylase (Sommerville *et al.*, 1998). Its primary function would be to deacetylate the core histones incorporated into newly-synthesized chromatin during the rapid cell cycles leading to blastula formation. A major event in the assembly and accumulation of the HD complex is the translocation of the HDm protein into the nucleus during oogenesis. Here we examine the features of HDm that are responsible for its nuclear uptake, identifying the charged carboxy-tail domain as a target for modification by phosphorylation.

## Results

### *Overexpression of HDm in growing oocytes leads to nuclear uptake and chromatin binding*

The *Xenopus* maternally-expressed protein HDm (Ladomery *et al.*, 1997) has been shown to be a marker for histone deacetylase (HD) activity during early development. This protein is a component of a 360 kDa complex that is accumulated in the nuclei of growing oocytes (Sommerville *et al.*, 1998). One feature of HDm, also found in the homologues human HD1 (Taunton *et al.*, 1996) and *Drosophila* Rpd3 (De Rubertis *et al.*, 1996), is the presence of the charged C-terminal domain which in the *Xenopus* protein contains 35% acidic (D + E) residues and 23% basic (K + R) residues (Figure 1). It is noted that, within this domain, HDm has a sequence 441**KR**VKTEEEKEGED**KK**DV**K**458, similar to the bipartite nuclear localization signal (NLS, Dingwall and Laskey, 1991) 534**KR**KTEEESPLKD**K**DA**KK**551 which is contained in the *Xenopus* nuclear protein N1/N2. This sequence similarity may have some significance because the two proteins have related functions: whereas HDm is believed to deacetylate histones H3 and H4, N1/N2 acts as a chaperone for diacetylated H3 and H4 awaiting incorporation into chromatin (Kleinschmidt and Seiter, 1988).

Requirement of the tail domain for import of HDm into the nucleus can be tested *in vivo* by injecting radiolabelled fragments into the cytoplasm and recording subsequent levels in isolated nuclei. Injection of $^{35}$S-labelled HDm protein (Figure 2A) into the cytoplasm of *Xenopus* oocytes results in a 10-fold concentration of the protein in the nucleus after 24 h (Figure 2B). In comparison, a truncated polypeptide (ΔH, Figure 1A) which lacks the charged tail domain, fails to be concentrated substantially in the nucleus (Figure 1B). The kinetics of nuclear import of HDm are similar to those reported for nucleoplasmin (Vancurova *et al.* 1995), the efficient transport of which appears to be dependent on phosphorylation by an associated casein kinase II (CK2) activity. HDm has six potential CK2 phosphorylation sites, five of which are located within the charged tail domain (Figure 1).

One advantage of the effectiveness of HDm uptake into oocyte nuclei is that the subsequent state of the chromatin can be examined directly. The lampbrush chromosomes of *Xenopus* oocytes are poorly immunostained with antibodies raised against the C-terminal domain (anti-ΔV). Previous studies showed only a low level of turnover of acetyl groups on histone H4 of lampbrush chromosomes (Sommerville et al., 1993) and also in the maternal pool of histones stored in *Xenopus* oocytes (Almouzni et al., 1994), indicating that endogenous histone deacetylase activity is maintained at a low level in oocytes. At 24h after injection of radiolabelled HDm into the cytoplasm of actively transcribing (stage III) oocytes, nuclear

immunostaining with anti-ΔV is greatly increased. In addition to *de novo* immunostaining of the chromosomes, the chromosomes are seen to be condensed (foreshortened, Figure 3A,B) compared with injection of unrelated protein (the mRNA-associated DEAD-box helicase p54, Figure 3C). Furthermore a high background immunostaining of nucleoplasm indicates high-level loading of the nucleus with HDm (Figure 3B). From measurement of relative radiolabelling, it can be calculated that approximately 10 pg of HDm is imported into the nucleus under the conditions described, a value approaching the total mass of histone in the chromatin. Incubation of injected oocytes in the presence of 10 mM sodium butyrate or 5 ng/ml trichostatin A (TSA, both potent inhibitors of deacetylase activity) does not inhibit uptake of HDm into the nucleus and chromatin binding, but does prevent chromosome condensation (Figure 3D). Thus, injection of complete molecules of HDm into living oocytes results in their import into the nucleus, binding to the chromatin and gross changes in the structure of the chromatin. We cannot tell, from these experiments alone, how specific are the effects: particularly to what extent HDm might be targeted to particular sites on the chromatin and to whether HDm is actually deacetylating histones in this *in vivo* situation. However, it is noted that immunostaining tends to be punctate rather than evenly spread along the chromosome axis (Figure 3B) and that not all lateral loops on the condensed lampbrush chromosomes are retracted, indicating that transcription at some loci is continuing after exposure to HDm. Also, isolation of chromosomes at 8h after injection of HDm reveals chromosomes with less evidence of length contraction and loop retraction but which, nevertheless, immunostain clearly with anti-ΔV (not shown).

***The charged tail domain is phosphorylated at selected sites by the nuclear protein kinase CK2***

HDm contains six possible sites for phosphorylation by CK2 but no obvious sites for phosphorylation by cell cycle-dependent kinases. Five of the six CK2 sites are located in the charged tail domain and are represented in GST fusion proteins as shown in Figure 4A. CK2 activity is readily available from oocyte nuclei (germinal vesicles) isolated under oil and this activity can be separated from most of the nuclear protein by affinity binding to heparin-Sepharose (A.J.L. and J.S., unpublished). Nevertheless, the presence of nuclear proteins provides internal markers for the phosphorylation reactions, the histone H3/H4 chaperone N1/N2 (running at 110/105 kDa on SDS-PAGE) and the histone H2A/H2B chaperone nucleoplasmin (running at 30 kDa on SDS-PAGE) being particularly appropriate. Incubation of the different fusion proteins with nuclear extract and $^{32}$PγATP shows that only proteins containing the charged tail domain (ΔR and ΔV) are

phospholabelled: proteins containing only the amino-terminal CK2 site are never phospholabelled (Figure 4B). The GST-ΔV protein undergoes limited proteolysis if expressed in *E. coli* K strains, resulting in products running on SDS-PAGE with apparent masses of 43 kDa and 39 kDa due to polypeptide cleaved from the carboxyl end. Since the 43 kD product phosphorylates almost as efficiently as complete GST-ΔV, it can be concluded that accessible sites are located beyond 5 kDa from the carboxyl terminus. However, the 39 kDa product is much less efficiently phospholabelled, indicating rare availability of sites located beyond 9 kDa from the carboxyl terminus. Little difference is seen in phospholabelling patterns of the GST fusion propteins using either whole nuclei or CK2 activity isolated from the nuclei (Figure 4B), indicating that the activity of CK2 is sufficient to account for the phosphorylation of HDm observed in nuclear extracts. As predicted, the CK2 activity is efficiently inhibited by low concentrations of heparin and is also specific for the mRNA-binding proteins FRGY2a/b (Figure 4B).

A more accurate fix on the phospholabelled sites is obtained from analysing peptides, produced by trypsin digestion of full-length phospholabelled GST-ΔV, on an amino acid sequencer. Two peaks of radioactivity were obtained by separation of the digest on fine-bore HPLC (Figure 5A). Peptides from the peak fractions were then sequenced and material from each cycle was collected on filters and assayed for radioactivity. In peptide 1 (Figure 5B), radioactivity was recovered only in cycle 22, indicating that the serine residue at position 22, but not the serine residue at position 17 (which is another of the potential CK2 sites), is phosphorylated. In peptide 2 (Figure 5C), peaks of radioactivity were recovered from cycles 8 and 10, corresponding to the two serine residues present at these positions. These same peaks of radioactivity were found in two separate sequencing runs. No evidence was found of a peptide containing the sequence TEEE in a labelled form, which tends to discount the CK2 site that is located within the putative NLS. From these results, it appears that phosphorylation of HDm, *in vitro*, is restricted to three out of six possible sites.

***Phosphorylated isoforms of HDm are present in the nuclei of full-grown oocytes***

On closer examination of immunobots of proteins extracted from the nuclei of stage VI oocytes and separated by SDS-PAGE, it is seen that the immunoreactive band is composed of several closely-arrayed sub-bands. That these sub-bands represent diffently charged isoforms is indicated by isoelectrofocussing (not shown) and that the isoforms result from differential phosphorylation is confirmed by treatment with alkaline phosphatase, which results in the resolution of the immunostaining material as a single, sharp band (Figure 8). At least four distinct isoforms can be discriminated, and it is reasonable to assume that they correspond to non-

phosphorylated HDm plus phosphorylation at one, two and three of the sites described in the last section. The only other stage of development which exhibits such multiple isoforms is blastula (not shown), during which stage of development most of the histone deacetylation is believed to occur.

***Nuclear uptake of fusion protein is dependend on the C-terminal region of HDm containing the putative NLS***

On injecting phospholabelled GST-ΔV into the cytoplasm of full-grown (stage VI) oocytes, the kinetics of nuclear uptake can be recorded by SDS-PAGE/autoradiography of the contents of nuclei isolated after various time intervals. Of the various forms of GST-ΔV injected, only the protein with a complete carboxyl terminus is accumulated in the nucleus (Figure 6A): the 42 kDa (-5) and 37 kDa truncated forms are not detected in the nucleus and are eventually (over 50 h) dephosphorylated and/or degraded in the cytoplasm. The kinetics of nuclear uptake of GST-ΔV (Figure 6B) show a rapid and efficient translocation: over 80% of the protein is isolated with the nucleus 10 h after injection. Total protein uptake in these experiments amounts to more than 1 ng/oocyte, which compares with a synthetic rate of core histones in full-grown oocytes of 0.5 ng/h (Adamson and Woodland, 1974) (check histone/NP values)

***Inhibition of CK2 activity blocks nuclear uptake of the tail domain***

Injection of phosphorylated and dephosphorylated GST-ΔV into oocytes treated with the inhibitor of CK2, quercetin and its inactive analogue quercetin 3β-D-rutinoside (rutin).

***Phosphorylation of the tail domain enhances interaction with importin α***

***Nuclear HDm is dephosphorylated during progesterone-induced maturation of stage VI oocytes***

The multiple bands revealed on immunstaining blots of stage VI nuclear protein undergo change within the first few hours of treating the oocytes with progesterone. Progesterone induces oocyte maturation, which is characterized by many changes including chromatin condensation, hyperphosphorylation of the nuclear lamins and nuclear breakdown (GVB). However, in the few hours preceding GVB, nuclei can still be isolated and immunoblotting of their proteins shows a time-dependent resolution of multiple HDm bands into the single, fastest migrating, band. It is likely that this change is due to the activation of a nuclear phosphatase.

## Discussion

### *Role of phosphorylation in HD nuclear uptake*

Import of a protein into the nucleus is generally dependent upon the presence of a nuclear localization signal (NLS). The NLS can take the form of either a block of mainly basic residues, the model being the sequence 126PKKKRVK132 that occurs in SV40 T-ag, or a bipartite motif in which two short basic regions are separated by ten unspecified residues (Dingwall and Laskey, 1991). The function of the NLS can be regulated, however, by phosphorylation of proximal sites, resulting in either acceleration or inhibition of nuclear uptake of the protein. For instance, the rate of NLS-dependent nuclear import of T-ag is increased 40-fold by phosphorylation of $Ser^{112}$ by CK2, whereas phosphorylation of $Thr^{124}$ by cdc2 inhibits nuclear import (Rhis *et al.*, 1991; Jans and Jans, 1994). Phosphorylation of such residues in the vicinity of the NLS acts to regulate import through modulation of protein-protein interactions.

From the microinjection experiments in this report, we show the carboxy-terminal region of ~40 amino acid residues to be essential for efficient nuclear uptake of HDm. Within this region, we tentatively identify the sequence 438**KKVKRVK**TEEEKEGED**KKD**V**K**458 as being important for nuclear uptake. Either the first seven residues, or a bipartite grouping of residues 441-442 and 454-458 could act as an NLS. It is interesting to note that a very similar sequence 531LV**RKKRK**TEEESPLKD**K**DA**KK**551 has been been identified as the NLS in the *Xenopus* nuclear protein N1/N2, originally as the first seven residues (Kleinschmidt and Seiter, 1986) and latterly as a bipartite grouping of residues 534-535 and 545-549 (Dingwall and Laskey, 1991). HDm and N1/N2 have related functions in the utilization of histones during the early development of *Xenopus*. During oogenesis, there is accumulation of a large pool of histones ( ng/cell; Adamson and Woodland, 1974), sufficient for assembly of much of the new chromatin through the 13-14 rapid cell divisions of early embryogenesis. The stored histones exist in oocytes in association with specific protein chaperones which together form soluble complexes: histone H3/H4 coupled with N1/N2 to form a complex sedimenting at 5S and a histone H2A/H2B coupled with nucleoplasmin to form a complex sedimenting at 7S (Kleinschmidt *et al.*, 1985). However, these stored core histones are accumulated as acetylated forms: non-nucleosomal histone H4, for example, has been shown to be diacetylated, not only in *Xenopus* oocytes and early embryos (Almouzni *et al.*, 1994), but also during the early development of sea urchins (Chambers and Shaw, 1987) and starfish (Ikegami *et al.*, 1993). Once the acetylated histones are incorporated into chromatin, the new nucleosomal structures are stabilized by histone deacetylation. HDm is believed to be the catalytic agent involved in developmentally-regulated

deacetylation in *Xenopus* (Ladomery *et al.*, 1997). Histone deacetylase activity is the property of a 360 kDa protein complex containing HDm, which has been shown to deacetylate peptides representing the amino-terminal region of histone H4, in addition to mixtures of acetylated core histones (Sommerville *et al.*, 1998). The HDm complex and N1/N2 similarly interact with core histones in the nucleus and so might be expected to share regulatory features.

Nuclear import of the other histone chaperone, nucleoplasmin, has been shown to be regulated by phosphorylation, particularly phosphorylation of sites in the vicinity of the NLS by CK2 (Vacurova *et al.*, 1995). Of the six potential sites in HDm for phosphorylation by CK2, we have shown in this report that three are phosphorylated *in vitro* by a form of CK2 isolated from *Xenopus* oocyte nuclei (germinal vesicles). These three sites are occupied by serine residues which all lie within the charged tail domain, at positions -15, -17 and -45 from the start of the putative NLS. The most proximal two sites are more efficiently phosphorylated than the distal site and have a location relative to the putative NLS similar to the CK2 sites (at positions -14 and -15) whose phosphorylation enhances the rate of nuclear import of T-ag (Rhis *et al.*, 1991; Jans and Jans, 1994). In comparison, the CK2 sites in nucleoplasmin, most likely to be used in NLS-dependent regulation, are located at positions +7 and +8 beyond the end of the NLS.

One potential CK2 site in HDm that we did not detect as a phospholabelled peptide is 145TEEE148, which lies within the putative bipartite NLS. This same sequeence occupies an identical position within the bipartite NLS of N1/N2. However, it is still possible that this site plays a regulatory role in HDm: it may be phosphorylated under special conditions not met in the *in vitro* labelling procedure used here.

That phosphorylation of HDm is actually occuring during import into oocyte nuclei *in vivo*, is indicated by the separation, on SDS-PAGE, of multiple immunostaining bands. The multiple banding is seen in protein samples taken from isolated nuclei, but not from isolated cytoplasms (Sommerville *et al.*, 1998) and we show here that at least four components can be resolved to a single band after treatment with alkaline phosphatase. Although the number of slower migrating bands corresponds to the number of phospholabelled residues detected after *in vitro* phosphorylation, we do not know if the same sites are being used *in vivo*. The presence of multiple HDm bands in the nuclei of full-grown (stage VI) oocytes and their rapid resolution to a single band after treatment of the oocytes with progesterone, indicates that activity of phosphatases is low prior to induction of maturation, but that, within a few hours, activity becomes high enough to completely dephosphorylate the stored HDm before GVBD. It should be noted that the pool of HDm stored in the nucleus is not distributed throughout the nucleoplasm but rather is

associated with the nuclear envelope: immunostaining of sectioned oocytes and cleavage stage embryos shows a concentration of signal around the inside of the nuclear membrane. There have been previous reports that HD activity is associated with nuclear matrix material in immature chicken erythrocytes (Li *et al.*, 1997).

That the stored HDm in non-injected oocytes is not associated with the endogenous chromatin is shown by examination of isolated lampbrush chromosomes, which are not immunostained by the same antibodies that immunostain chromosomes from oocytes injected with an excess of *in vitro*-synthesized HDm. Over-supply of synthetic HDm apparently brings about a release from the constrains which anchor the endogenous HDm on the nuclear envelope. A secondary effect of the binding of injected HDm to the chromosomes is that it causes premature condensation of the chromosomes. However, we do not know if this effect is due to targeting of histone deacetylase activity to the chromatin or to some other, less-specific, cause. Nevertheless, we do note that such condensation is blocked by low concentrations of trichostatin A (TSA), which is a specific inhibitor of HDm-associated histone deacetylase activity (Sommerville *et al.*, 1998).

Phosphorylation of HDm may be important in respects other than regulation of nuclear import. We have shown previously that treatment of extracts containing the 360 kDa histone deacetylase complex with alkaline phosphatase leads to a loss of enzyme activity (Sommerville *et al.*, 1998). In addition to the apparently phosphorylated forms of HDm seen in nuclear extracts from stage VI oocytes, similar slower migrating forms are seen in extracts from embryos at midblastula. Changes in the level of phosphorylation of *Xenopus* nucleoplasmin have been reported to occur at oocyte maturation, when slower migrating forms appear, and just after the midblastula transition, when they appears to be a dephosphorylated (Burglin *et al.*, 1987). The reason for maintaining nucleoplasmin in a phosphorylated state through early embryogenesis is not altogether clear, although this covers the period of maximum release of histones to be assembled into new chromatin.

No observations have been made on the mechanism of nuclear uptake of other histone deacetylases, therefore we do not know to what extent our results will apply to other cell types. It is most likely that HDm is primarily a chromatin deposition deacetylase since it is expressed only during oogenesis and early embryogenesis (Ladomery et al., 1997), whereas the histone deacetylases in yeast (Vidal and Gaber, 1991; Rundlett *et al.*, 1996), *Drosophila* (De Rubertis et al, 1996) and mammalian somatic cells (reviewed, Wollfe, 1997) have been ascribed functions in the regulation of gene transcription. Nevertheless, there does appear to be some conservation of features in the tail domain that we have identified as having regulatory functions in HDm (Figure 9).

## Materials and Methods

### *Proteins and antibodies*

HDm and polypeptide fragments were synthesized either as glutathione S-transferase (GST) fusions, or from run-off transcripts translated in rabbit reticulocyte lysates (RL). GST fusion proteins were expressed from pGEM (Pharmacia) vectors and isolated on glutathione-Sepharose 4B according to the manufacturer's instructions. Antibodies to DR/DH and DV fusion proteins were raised in rabbits as were antibodies directed against a synthetic peptide representing the carboxy-terminal seventeen amino acid residues of HDm (anti-Cpep). Reticulocyte lysate translation products were synthesized in the TNT (Promega) system for 2 h at 30°C in the presence of 10 mCi/ml of $^{35}$S-methionine (1,000 Ci/mmol, Amersham). Transcription was from either the complete cDNA sequence in pBlueScript (Promega) or from 3' truncations extending into the coding region. Labelled protein was obtained from Sephadex-G50 spin column fractions .

### *Oocyte extracts*

Ovary fragments were gently mixed for 1-2 h (until the tissue had disaggregated) in calcium-free OR-2 medium () containing 2 mg/ml of collagenase (Sigma, Type I). Defolliculated oocytes were washed through several changes of calcium-containing medium and allowed to recover for 16-24 h before being used. Pools of 50 oocytes (stages I-IV) or 25 oocytes (stages V and VI) were collected and homogenized by cycling twenty times through a pipette tip in three volumes of homogenization buffer: 0.1 M KCl; 2 mM $MgCl_2$; 2 mM dithiothreitol; 0.2% Nonidet P-40; 20mM Tris-HCl, pH 7.5. Ater centrifugation at 10,000 rpm for 15 min in a swing-out rotor (Sorvall SW24), the clarified supernatant (SN10) was carefully removed. Nuclei and cytoplasms were isolated under paraffin oil as described previously (Paine *et al.*, 1992) and were resuspended in either homogenization buffer or protein kinase buffer (see below).

### *Immunostaining*

Total protein minus pigment and yolk equivalent to three oocytes or embryos was separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were incubated for 2 h at 20°C with either anti-DV, anti-DR/DH or anti-Cpep (1:2000) then with peroxidase-conjugated anti-rabbit IgG (1:3000, Chemicon) and developed with 3,3'diaminobenzidine/$H_2O_2$.

Protein transfers were also developed using the ECL (Amersham International) procedure, after reaction with anti-Cpep (1:5000) and peroxidase-conjugated anti-rabbit IgG (1:10,000)

Fragments of ovary dissected from immature *Xenopus* were fixed for 1 h in 3% paraformaldehyde/0.25% glutaraldehyde/0.1 M phosphate buffer, pH 7.2, and wax-

embedded and sectioned as described (Kelly *et al.*, 1991). Dewaxed sections were immunostained with anti-DR/DH (1:200) followed by FITC-conjugated anti-rabbit IgG (1:500, Chemicon) as described previously ().

***Protein phosphorylation***

About 1mg of the GST-DR, GST-DR/DH and GST-DV fusion proteins were added to extracts equivalent to a single nucleus (GV) from stage VI oocytes or from an equivalent amount of CK2 isolated from nuclear extracts by chromatography on heparin-Sepharose (Pharmacia) . After incubation in the presence of $^{32}$PgATP at 20°C for 30 min, samples were run on SDS-PAGE gels and analysed by autoradiography.

***Identification of phosphorylated residues***

Phospholabelled GST-DV fusion protein was digested with trypsin (20mg/ml) in 0.1 M ammonium bicarbonate at 37°C for 4 h. After lyophilization, the sample was raised in 50ml of 0.1% trifluoroacetic acid and 20ml of this was applied to a C-18 microbore column. The elution gradient used was a 5 min isocrat step of 5% acetonitrile, followed by a gradient to 50% acetonitrile over 30 min and a 10 min rise to 95%. Fractions were collected every min (80ml) and 2ml aliquots were spotted on to filter paper and assayed overnight for radioactivity using a phosphorimager. Peak radioactive fractions were lyophilized and resuspended in 10ml of 30% acetonitrile. These samples were spotted on to a half disk of Sequelon-AA (Millipore) membrane (PVDF derivitised with aryl amine groups) and dried at 55°C on a heating block. The dried peptide was then attached to the membrane by addition of 5ml of a 10mg/ml solution of carbodiimide (EDC). After leaving at room temperature for 20 min, the membrane was washed three times in 0.5ml of 50% methanol and then placed in the sequencer. The extracted ATZ derivative from each sequencing cycle was collected, dried and resuspended in 10 ml of 90% methanol. Aliquots of 2ml were spotted on to filter paper and assayed overnight for radioactivity using the phosphorimager.

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**Figure legends**

**Fig. 1.** Sequence motifs present in the charged carboxy-terminal domain of HDm.

**Fig. 2.** HDm uptake into oocyte nuclei and binding to chromatin. (A) Autoradiograph showing that HDm and the DH fragment are substantially the only labelled proteins synthesized in the reticulocyte lysate system. (B) Injection of labelled proteins into the cytoplasm of stage V oocytes results in higher levels of nuclear accumulation of HDm (filled circles) than DH (open squares). (C-F) Immunostaining with anti-DV of lampbrush chromosomes from stage III oocytes : phase (C) and fluorescent (D) images of a condensed bivalent chromosome produced 24h after injection with HDm (the limits of the bivalent are indicated by arrows); (E) fluorescent image of (non-condensed) bivalent after injection with a non-HDm sample (bivalent runs across the field, beyond the arrows); (F) fluorescent image of telomeric region (T) 24 h after injection with HDm and incubation in 5 ng/ml trichostatin (TSA). Bar represents 10mm.

**Fig. 3.** Phosphorylation of fusion proteins containing different regions of HDm by CK2 activity from oocyte nuclei.

**Fig. 4.** Mapping of phosphorylation sites in HDm.

**Fig. 5.** Dependence on the carboxy-terminal region of HDm for nuclear uptake.

**Fig. 6.** Requirement of tail-domain phosphorylation for nuclear import and binding to importin $\alpha$.

**Fig. 7.** Dephosphorylation of nuclear HDm during oocyte maturation.

**Fig. 8.** Sequence comparison of tail domains of histone deacetylases from higher eukaryotes.

FIG. 3 Uptake of HDm into the nucleus and chromatin binding.
METHODS: Oocytes were microinjected with 10-20 nl samples of HDm extract and incubated at 20°C for the time indicated. Germinal vesicles were isolated under oil as described[21] and lampbrush chromosomes were prepared as described previously[12].

# Histone deacetylase activity is accumulated in *Xenopus* oocytes as protein complexes and is transported to the nucleus prior to maturation

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Running title: *Histone deacetylase in oocytes*

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**Summary**

**Reversible acetylation of core histones plays an important regulatory role in transcription and replication of chromatin. The acetylation status of chromatin is determined by the equilibrium between activities of histone acetyltransferases and histone deacetylases (HDs). The *Xenopus* protein HDm shows sequence homology to other putative histone deacetylases, but its mRNA is expressed only during early development. Both HDm protein and acetylated non-chromosomal histones are accumulated in developing oocytes, indicating that the key components for histone deposition into new chromatin during blastula formation are in place by the end of oogenesis. Here we show that the main, 57 kDa, form of HDm undergoes steady accumulation in the nucleus where it is organized in a multiprotein complex of ~360 kDa. This complex has HD activity that is sensitive to trichostatin A, zinc ions and phosphatase treatment. The 57 kDa HDm protein is a marker for HD activity throughout oogenesis and early embryogenesis. An earlier-expressed 63 kDa protein, which is exclusively cytoplasmic and associated with membrane fractions, may be a precursor of the 57 kDa form that it is modified for membrane attachment. Reasons for redistribution of HDm during early development are discussed.**

*Abbreviations*: AP, alkaline phosphatase; GST, glutathione-S-transferase; HAT, histone acetyltransferase; HD, histone deacetylase; TSA, trichostatin A.

**Introduction**

Early embryogenesis in *Xenopus* proceeds from a fertilized egg to a blastula of ~10,000 cells in a series of 13-14 rapid cell divisions. Throughout this period there is no transcriptional activity and the assembly of new chromatin from almost continuously replicating DNA is largely dependent on a maternal pool of histones and assembly factors. In general, chromatin assembly involves the association of pre-acetylated core histones with replicating DNA and subsequent stabilization of nucleosomes by histone deacetylation. Newly-synthesized histone H4 is acetylated at defined lysine residues by a cytoplasmic histone acetyltransferase (HAT B; Sobel et al., 1994). Sequencing of the amino-terminus of newly-synthesized histone H4 from a range of different organisms has shown it to be diacetylated, at lysine residues 5 and 12 (Sobel et al., 1995), although yeast HAT B activity on histone H4 in vitro shows some variation from this pattern (Kleff et al., 1995; Parthun et al., 1996). Deacetylation would normally occur soon after histone deposition in new chromatin but information is only now becoming available as to how histones are accessed by histone deacetylase (HD) and to how the deacetylation reaction is regulated.

The aspect of histone deacetylation that has received most attention recently is its role in the regulation, most often repression, of the activity of specific sets of genes (reviewed by Wolffe, 1996, 1997). For instance, association of HD1 with the adaptor protein RbAp48 (Taunton et al., 1996), or with the corepressor of the mammalian Mad/Max complex, Sin3 (Alland et al., 1997), or with N-CoR, the corepressor of the thyroid hormone receptor (Heinzel et al., 1997), may serve to target the deacetylase complex to specific chromatin sites. Such interactions appear to be restricted to modulation of transcriptional activity of specific genes recognized though the presence of transcription factors, such as Mad-Max and the thyroid hormone receptor, localized to their promoters. The more extensive deacetylation reactions occurring after replication may involve alternative components. Two, chromatographically-separable, multiprotein complexes containing histone deacetylases, designated HDA and HDB, have been described in yeast (Carmen et al., 1996; Rundlett et al., 1996) but, apart from the deacetylases themselves, other protein components have not been identified.

*Xenopus AB21* (data bank accession number: X78454) was initially recognized as a homologue of the yeast gene regulator *RPD3* (Vidal and Gabor, 1991) which was later shown also to be a homologue of human *HD1* (Taunton et al., 1996). There has now been cloned and identified an additional series of putative histone deacetylases - in yeast (Rundlett et al., 1996), *Caenorhabditis* (Waterston et al., 1992), *Drosophila* (De Rubertis et al., 1996), and mouse (Bartl et al., 1997) - all of which appear to have a conserved region stretching from near the amino terminus to more than half-

way through the protein sequence. It is likely that this conserved region represents the enzyme core responsible for deacetylase activity (Ladomery et al., 1997).

The mRNA encoded by *Xenopus AB21* is expressed only in oocytes, remains stable in embryos up to neurula and has sequence characteristics of a maternal message. Its protein product, HDm (maternal histone deacetylase; Ladomery et al., 1997), is synthesized during oogenesis and decreases in amount after mid-blastula, when the cell-cycle slows down to a normal rate and cells start to differentiate. Here we show that HDm is a marker for HD activity during early development and describe its presence in protein complexes and the timing of its transportation into the nucleus.

## Materials and methods

*Proteins and Antibodies*

Glutathione S-transferase (GST) fusion proteins were expressed from pGEM (Pharmacia) vectors and isolated on glutathione-Sepharose 4B according to the manufacturer's instructions. The fragments of HDm cDNA used in the constructs have been described (Ladomery et al., 1997). Antibodies to ΔR/ΔH and ΔV fusion proteins were raised in rabbits as were antibodies directed against a synthetic peptide representing the carboxy-terminal sixteen amino acid residues of HDm plus an additional N-terminal cysteine (anti-Cpep).

*Oocyte Extracts*

Ovary fragments were gently mixed for 1-2 hours (until the tissue had disaggregated) in calcium-free OR-2 medium (Evans and Kay, 1991) containing 2 mg/ml of collagenase (Sigma, Type I). Defolliculated oocytes were washed through several changes of calcium-containing medium and allowed to recover for 16-24 hours before being used. Oocytes were sorted into individual stages according to Dumont (1977). Pools of 50 oocytes (stages I-IV) or 25 oocytes (stages V and VI) were collected and homogenized by cycling twenty times through a pipette tip in three volumes of homogenization buffer: 0.1 M KCl; 2 mM $MgCl_2$; 2 mM dithiothreitol; 20mM Tris-HCl, pH 7.5. In some early experiments, the homogenization buffer was adjusted to 0.2% Nonidet-P40. After centrifugation at 10,000 rpm for 15 minutes in a swing-out rotor (Sorvall SW24), the clarified supernatant (SN10) was carefully removed. Alternatively, extraction was performed using the organic solvent 1,1,2-trichlorotrifluoroethane (Freon, Sigma) as described (Evans and Kay, 1991). Partitioning using Triton X-114 was carried out as described previously (Hancock et al., 1989). Nuclei and cytoplasms were isolated under paraffin oil as described

previously (Paine et al., 1992) and were resuspended in homogenization buffer for further extraction.

*Immunostaining*

Total protein, minus pigment and yolk, equivalent to two oocytes, embryos or cytoplasms or to ten nuclei, was separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were incubated for 2 hours at 20°C with IgG (0.2-1.0 μg/ml) isolated from either anti-ΔV, anti-ΔR/ΔH or anti-Cpep antiserum, then with peroxidase-conjugated anti-rabbit IgG (1:10,000, Chemicon) and developed using the ECL (Amersham International) procedure.

Fragments of ovary dissected from immature *Xenopus* were fixed for 1 h in either 3% trichloroacetic acid or 3% paraformaldehyde/0.25% glutaraldehyde/0.1 M phosphate buffer, pH 7.2, and wax-embedded and sectioned as described (Kelly et al., 1991). Dewaxed sections were immunostained with anti-ΔR/ΔH (1:200) followed by FITC-conjugated anti-rabbit IgG (1:500, Chemicon).

*Enzyme Assay*

Deacetylase substrates were prepared essentially as described by Sendra et al.(1988). Purified rat liver histones (2 mg) or a peptide corresponding to the eighteen amino-terminal residues of histone H4 (1 mg) were dissolved in 0.5 ml of 50 mM Na borate pH 9.0 and mixed with 6 mCi of $^3$H-acetic anhydride (Amersham, 8.9 Ci/mmole, 12 mCi/ml in dioxane). After 3 hours at 0°C the mixture was adjusted to 0.25 M HCl and the peptides precipitated with 12 vol. acetone, washed three times in cold acetone and dried under vacuum. Histone deacetylase activity was assayed according to Li et al. (1996). Up to 100 μl of nuclear extract was mixed with 150 μl of assay buffer (25 mM Na phosphate/citric acid pH 7.0), 20 μl of $^3$H-acetylated peptide or histone mix (about 1.2 x $10^6$ dpm, dissolved in assay buffer) and $dH_2O$ up to a total volume of 300 μl. After 1 hour at 37°C the reaction was stopped by adding acetic acid and HCl to 0.12 M and 0.72 M respectively followed by 2 ml of ethyl acetate (Sigma). Samples were then vortexed and centrifuged at 9000 x g for 1 minute. Half the volume of ethyl acetate was removed and the dissolved $^3$H-acetate was measured by scintillation counting. Samples were dephosphorylated by adding 2 U of alkaline phosphatase (Sigma, type III) to 2.5 μl of embryo extract, or 20 μl of oocyte extract, adjusting to pH 8.3, and incubating for 1hour at 22°C prior to assay.

*Gradient Analysis*

Clarified supernatants (SN10 fractions) were layered on 10-25% glycerol gradients made up in: 0.1 M KCl; 2 mM $MgCl_2$; 2 mM dithiothreitol; 0.2% Nonidet P-40; 20mM Tris-HCl, pH 7.5. After centrifugation at 30,000 or 36,000 rpm in a 6 x 5 ml

swing-out rotor (Beckman SW50Ti) at 0°C for 16 hours, the tube contents were fractionated. Fractions were analysed for HDm (by immunoblotting) and HD activity (by the in vitro assay). Size was calculated using protein standards in parallel gradients: haemoglobin (67 kDa); IgG (160 kDa); apoferritin (443 kDa); IgM (960 kDa).

## Results

*HDm and Potential Histone Deacetylase Activity are Accumulated through Oogenesis*

The maternal histone deacetylase, HDm, consists of a region highly conserved between various putative deacetylases (Ladomery et al., 1997) plus a more variable region terminating with a sequence of 100 residues rich in charged side chains (Fig. 1). It is hypothesized that the conserved region represents the enzyme core and the variable region contains regulatory sequences which may be specific to the particular deacetylase. Antibodies were raised against GST-HDm fusion proteins containing the conserved region (anti-ΔR/ΔH), the charged tail domain (anti-ΔV) and against a synthetic peptide representing the carboxy-terminal sixteen amino acid residues of HDm (anti-Cpep). All three antibodies recognize a protein of 57 kD present in extracts from *Xenopus* oocytes and embryos separated by SDS-PAGE.

The immunoblotting profiles were compared with an *in vitro* assay (Li et al., 1996) to monitor levels of histone deacetylase (HD) activity in the same oocyte and embryo extracts. As can be seen (Fig. 2A), the amount of soluble HDm per oocyte increases steadily through the course of oogenesis, to reach a peak in full-grown (stage VI) oocytes. It was shown previously that this amount of immunoreactive protein remains fairly constant through oocyte maturation (M), fertilization and the rapid cell cleavage stages of early embryogenesis, but decreases after blastula (Ladomery et al., 1997). Measurement of HD activity in the same samples (Fig. 2C) shows that the profile of activity through oogenesis and early embryogenesis is very similar to that of the HDm antigen itself. However, these values represent ~10,000-fold higher levels in oocytes compared to mid-blastula embryos on a per cell basis, emphasizing the degree of control over HD activity that must be exerted during oogenesis when the transcriptionally-active lambrush chromosomes maintain acetylated forms of histone H4 (Sommerville et al., 1993) and stored histone H4 is accumulated in a diacetylated state (Almounzi et al., 1994).

*HDm is Accumulated first in the Cytoplasm, then in the Nucleus, of Growing Oocytes*

Immunoblots, comparing the relative contents of soluble HDm in the cytoplasm and nucleus of oocytes at different stages of development (stage III - stage VI), show that HDm is present at much higher concentrations in the nucleus, indicating that the 57 kDa HDm protein undergoes immediate translocation into the nucleus after synthesis (Fig. 2B). In apparent contradiction to this picture of immediate nuclear accumulation, immunostaining of immature (stage I-II) oocytes in ovarian sections

shows that the highest concentrations of the HDm antigen are to be found in cytoplasmic structures at, or close to, the periphery of the oocyte (Fig. 3A). Similar distributions, in the form of vesicle-like structures, are detected using either anti-ΔV or anti-Cpep. Sections of larger (stage IV-V) oocytes show less immunostaining at the cell periphery and detectable amounts in the nucleus. However, nuclear staining is not distributed throughout the nucleoplasm, but rather is located at, or close to, the nuclear envelope. So even the nuclear form, which is readily extractable, appears to be restricted in its distribution and probably sequestered from the chromatin, which lies internally in oocyte nuclei.

The in situ staining of early oocytes highlights material which would be expected to sediment on low-speed centrifugation, and therefore to be lost from the oocyte extracts (SN10). Because of this, it was decided to extract the homogenates more thoroughly using the organic solvent Freon (Evans and Kay, 1991). In such extracts substantial amounts of an additional antigen of ~63 kDa were detected on immunoblots (Fig. 4A). Freon extraction of isolated nuclei and cytoplasms confirmed that most of the cytoplasmic signal in the earlier oocyte stages comes from the ~63kDa material (Fig. 4B). From further quantitation, it is estimated that in previtellogenic (stage I) oocytes more than 95% of the immunoreactive protein is cytoplasmic, whereas in full-grown (stage VI) oocytes more than 95% of the immunoreactive protein is nuclear.

On measuring HD activity in nuclear and cytoplasmic isolates after Freon extraction, it is seen that the nuclear material is much more active than the cytoplasmic material (Fig. 4C). Because cytoplasms not extracted with Freon ('soluble-fractions') contain up to 50% as much activity as do cytoplasms extracted with Freon (Fig. 4C), yet contain no detectable 63 kDa antigen (Fig. 2B), it can be concluded HD activity in cytoplasmic fractions derives from the small amounts of 57 kDa protein that they contain.

*Cytoplasmic HDm may be Modified by Acylation*

The finding that the cytoplasmic antigen pellets rapidly, is lost from cell extracts treated with non-ionic detergents such as Nonidet-P40 (not shown) and can be solubilized in Freon, is consistant with its modification by acylation. Acylated proteins, such as members of the ras family, can be separated from non-modified soluble forms by phase separation using the detergent Triton-X114 (Hancock et al., 1989). On applying this technique to oocyte extracts, it is seen on immunoblotting that almost all of the more slowly migrating, immunoreactive protein partitions into the detergent phase (Fig. 5A).The amino acid sequence of HDm shows no consensus at the amino terminus for modification by myristoylation, nor for prenylation at, or close to, the carboxy terminus: however, palmitoylation at internal cysteine residues

is a distinct possibility (Milligan et al., 1995). Since palmitoylation involves thioester linkage of the fatty acid to the protein, this modification can be distinguished on the basis of its susceptibility to cleavage by base such as hydroxylamine. Treatment of oocyte extracts with 0.5-2.0 M hydroxylamine results in reduction in amount of the ~63 kDa form, but not of 57 kDa form, as estimated by immunoblotting (Fig. 5B), indicating that the slower migrating immunoreactive components result from a modification of HDm compatible with palmitoylation. The conversion of the ~63 kDa immunoreactivity to protein at 57 kDa is clearly seen on comparing the effect of hydroxylamine with that of alkaline phosphatase, which does not change the original pattern of immunostained bands (Fig. 5C). That the 63 kDa protein is indeed closely related to the 57 kDa HDm protein, and not some fortuitously crossreacting component, is confirmed by positive immunoblotting with all three antibodies to the different regions of HDm (Fig. 5 C,D). Antibodies raised against non-related proteins, such as the α-subunits of protein kinase CK2, do not crossreact with the 63 kDa band (Fig. 5D).

*Characterization of HD Activity Present in Oocyte and Embryo Extracts*

Two types of HD activity found in yeast cells (Carmen et al., 1996) can be differentiated on the basis of sensitivity to the specific inhibitor trichostatin A (TSA, Ikegami et al., 1993). Whereas HDA is inhibited by 80% in the presence of 10 nM TSA, HDB is inhibited by less than 20% under the same assay conditions (Carmen et al., 1996). On titrating HD activity with increasing concentration of TSA, extracts from either oocytes or embryos show a single transition of sensitivity, developing ~80% inhibition at 0.5 ng/ml (~2 nM) TSA (Fig. 6A). Therefore the HD activity present in *Xenopus* oocytes and early embryos appears to be mainly, if not exclusively, of the HDA type.

It has been noted previously that yeast deacetylase is sensitive to the divalent cation $Zn^{2+}$ (Carmen et al., 1996). HDm activity in extracts from both oocytes and embryos is about 80% inhibited by 1mM $ZnCl_2$ (Fig. 6B). No similar effect is obtained with $Mg^{2+}$, indicating that excess zinc may be specifically disrupting the structure of HDm. A possible role of $Zn^{2+}$ in the structure of deacetylases has been suggested previously (Ladomery et al., 1997).

It has been reported previously (Brosch et al., 1992) that the substrate specificity of *Zea mays* deacetylase is influenced by treatment with alkaline phosphatase (AP). Treatment of oocyte and embryo extracts with 50 units/ml AP at 22°C for 60 min results in a loss of 60-90% of the HD activity in the in vitro assay (Fig. 6C). Endogenous HD activity in nuclear extracts was not increased by preincubation with ATP to a final concentration of 5 mM (not shown).

We have been unable to demonstrate significant HD activity in preparations of recombinant HDm expressed in bacteria as GST fusion proteins. This suggests that HDm must be modified in vivo to gain catalytic activity, possibly through association with other proteins or, in view of our results with AP, by phosphorylation. Attempts to increase the in vitro deacetylase activity of oocyte extracts, by adding various forms of recombinant HDm, were also unsuccessful. In fact, high concentrations of the GST-ΔR/ΔH protein (>1 mg/ml) actually reduced HD activity by up to 86% (results not shown). A possible explanation for this is that rHDm can sequester acetylated histones without cleaving them. If this proves to be the case, then the apparent presence of histone deacetylase-inhibiting activity in crude cell extracts (Carmen et al., 1996; D. A. W., unpublished results) could be due to the presence of free HD subunits.

*HDm is a Component of Multimolecular Complexes with Histone Deacetylase Activity*

Yeast HDA and HDB activities are associated with protein complexes of ~350 kDa and ~600 kDa, respectively (Carmen et al., 1996). The relationship between molecular size and deacetylase activity in *Xenopus* oocyte extracts was measured by rate-centrifugation analysis. Clarified supernatants were layered on linear 10-25% glycerol gradients, which were centrifuged until a 19S marker (IgM) approached, or reached, the bottom of the gradient and then fractionated. The fractions were analysed for HDm content (by immunoblotting) and histone deacetylase activity (using the in vitro assay).

Supernatants from previtellogenic (PV) ovary (containing only stage I oocytes) give a heterodisperse distribution of HDm activity, which extends from fractions corresponding to single proteins to complexes of ~360 kDa (Fig. 7A). Although stage I oocytes contain mainly the vesicle-associated 63 kDa form, the HD activity detected probably derives from the released 57 kDa form which shows up in the immunoblot (Fig. 7B). As described earlier, extraction with Freon gives a more efficient extraction of HDm proteins from early-stage oocytes. Sedimentation analysis of Freon-extracted PV ovary reveals not only a better recovery of the 63 kDa protein but also its sharper resolution into a single peak that sediments at a rate corresponding to a total mass of ~200 kDa (Fig. 7C). It is not known whether this inactive form of HDm is present as a multimeric protein in the membrane fraction or forms such a complex on its release from the membranes.

In comparison with non-extracted PV ovary samples, supernatants from non-extracted vitellogenic (stage V) oocyte homogenates give a more distinct peak of HD activity (Fig. 7A), which corresponds to those fractions showing peak immunoreactivity of the 57 kD protein (Fig. 7D). This peak would correspond to a protein complex with a total mass of ~360 kDa. Again, the 63 kDa cytoplasmic antigen is spread through much of the gradient but appears to contribute little to enzyme activity.

Supernatants from full-grown (stage VI) oocytes lack the 63 kDa protein and show HD activity sedimenting sharply at ~360 kDa (Fig 8A). A similar peak of activity is obtained from isolated nuclei (Fig. 8B). Immunoblot analysis of fractions from both nuclei (Fig. 8C) and cytoplasms (Fig. 8D) confirms that the 57 kDa protein in the ~360 kDa complex is mostly nuclear in origin. Pretreatment of stage VI oocytes with progesterone for 16 hours, which leads to the completion of oocyte maturation (including nuclear breakdown), has no significant effect on the sedimentation properties of the HD complex (Fig. 8A). Supernatants from Freon-extracted homogenates of early embryos (at mid-blastula) show that, in addition to the mature 57 kDa protein, there is a reappearance of the 63 kDa form (Fig. 8E). The

most likely explanation is that this reappearance is due to de novo protein synthesis and membrane-association of the putative precursor form. A second phase of translation would also explain the previously-noted observation that the mRNA encoding HDm persists as a stable maternal message through early development until neurula (Ladomery et al., 1997). Thus the pool of oocyte-synthesized HDm appears to be supplemented during blastula formation by further translation of its mRNA.

All of the results presented here indicate that the active form of histone deacetylase present in oocytes and early embryos consists of a protein complex of ~360 kDa which contains the 57 kDa HDm protein. To confirm that HDm-containing complex is associated with the HD activity detected in oocytes, immunoprecipitation experiments were carried out using anti-Cpep. Of the total amount of enzyme activity incubated with affinity selected IgG, 50% was recovered bound to protein A-Sepharose after thorough washing of the resin (Fig. 9). This compares with 0.6% of the activity bound to the resin in the absence of specific IgG. The affinity-bound fraction retained its activity while still coupled to the resin and, overall, about 95% of the input activity was recovered in the various fractions (Fig. 9), indicating the robust nature of the enzyme.

## Discussion

*Relationship between HDm proteins and HD Activity*

In all of the samples tested from *Xenopus* oocytes and early embryos, there is a direct correspondence between the amount of the 57 kDa HDm protein detected and the level of HD activity assayed. This relationship is apparent in a range of different situations, particularly in the developmental expression pattern and in sedimentation characteristics. The observation that over 50% of recovered HD activity is immunoprecipitated by antibodies directed against the carboxy-terminal peptide of HDm indicates that HDm represents the major HD activity present in oocytes. No other crossreacting bands are detected on immunoblotting soluble extracts from oocytes with antibodies directed against regions of HDm conserved in all other histone deacetylases described (Ladomery et al., 1997). However, extraction of oocyte homogenates or membrane fractions with detergents or organic solvent releases substantial amounts of additional immunoreactive protein with an apparent mass on SDS-PAGE of 63 kDa. The observations that the 63 kDa band partners the 57 kDa band in almost every situation of isolation, extraction and separation and that both bands crossreact with the three specific antibodies used in this study, point to a close relationship between the two proteins. The ability of hydroxylamine to covert the 63 kDa form to a 57 kDa form strongly indicates that the relationship is between a form modified by acylation (most likely palmitoylation) and its unmodified form.

The interpretation that the acylated form represents a cytoplasmic, membrane-associated precursor of the largely nuclear, unmodified form, gains support from the changes seen in their relative abundance with development. Immunoblotting shows progressive loss of the 63 kDa protein from the cytoplasm and progressive accumulation of the 57 kDa protein in the nucleus. The immunocytological studies can also be interpreted as showing translocation of HDm antigen from cytoplasm to nucleus.

*HD Complexes in Oocytes*

In several respects the HD complexes described here are similar to the HDA complexes described for *S. cerevisiae* (Carmen et al., 1996; Rundlett et al., 1996). In yeast, two distinct types of complex are found: HDA, which has an estimated mass of ~350 kDa, deacetylates all four core histones, and is strongly inhibited by TSA; and HDB, which has a mass of ~600 kDa and is relatively insensitive to TSA. All of the 57 kDa HDm antigen and nearly all of the HD activity recovered from both whole oocytes and oocyte nuclei, is found in a peak of material sedimenting with an average mass of 360 kDa. Although the membrane-released 63 kDa protein is also recovered as a protein complex (of ~200 kDa) we are unable to detect much HD activity in this material. No complexes were detected with masses approaching 600 kDa. Analysis of HD activity from oocyte extracts by gel-filtration (not shown) also gives an estimated mass between 350 and 400 kDa. The composition of the oocyte complex is not yet known, although crosslinking experiments indicate that they contain more than one molecule of HDm. Yet multimers of HDm alone may not be sufficient for HD activity, for none of the recombinant HDm proteins that have been expressed in bacteria have shown any activity. Work is in progress to identify other proteins in the ~360 kDa complex which might be required as cofactors for HD activity and to determine the role of phosphorylation in its control.

All of the samples extracted from oocytes and embryos show sensitivity to TSA comparable to that of yeast HDA, with 80% inhibition of HD activity at a concentration of 0.5 ng/ml (~2nM) TSA. Although substrate specificity has not yet been examined in detail, the conditions of in vitro deacetylation used in this present study show that a mixture of rat liver histones or a peptide corresponding to the amino-terminal eighteen amino acid residues of histone H4, act equally well as substrates for the oocyte enzyme.

*Sequestering of HD Activity during Oogenesis*

Accumulation of HDm-containing complexes, along with their associated HD activity, presents a problem for the oocyte in handling such enormous amounts of potential activity. A single full-grown oocyte contains as much HD activity as 10,000

embryonic cells at blastula. Yet oocytes contain chromatin, in the form of lampbrush chromosomes, that is highly active in transcription, a condition reflected by the widespread occurrence of hyperacetylated forms of histone H4. On immunostaining lampbrush chromosomes using antibodies which recognize specifically the four different acetylation sites in the amino-terminal region of histone H4 (Turner et al., 1992), three of the sites, Lys8, Lys12 and Lys16, were found to be acetylated at multiple foci within the chromatin (Sommerville et al., 1993). Furthermore, such extensive acetylation appeared to be stable, because incubation of the oocytes in the potent inhibitor of histone deacetylases, sodium butyrate, did not increase the signal given by the antibodies to acetylated lysines. Thus it appears that the HD activity accumulated in oocytes has little natural access to the endogenous chromatin. Indeed, lampbrush chromosomes isolated from stage III/IV oocytes are not immunostained using the anti-HDm antibodies (J. S., unpublished).

In addition to the acetylated histones contained in the chromatin, oocytes are accumulating large pools of acetylated histones to be used in the formation of new chromatin during the 13-14 cell divisions leading to blastula. It is estimated that each full-grown oocyte contains 21 ng of maternal histone H4 (Adamson and Woodland, 1974) which remains in a diacetylated form until after incorporation into the newly-replicated chromatin (Dimitrov et al., 1993; Almouzni et al., 1994). The storage of acetylated maternal histones and their rapid incorporation into new embryonic chromatin is a phenomenon that has been described in different developmental systems. For instance, in both sea urchins (Chambers and Shaw, 1984) and starfish (Ikegami et al., 1993) maternal histone H4 is stored in a diacetylated form which is followed, during blastula formation, by a wave of deacetylation. All of these observations serve to emphasize the need to keep apart stored HD activity and stored acetylated histones until after deposition in embryonic chromatin.

The observation that extracts taken from all stages of early *Xenopus* development have an immediately available histone deacetylase activity would argue that sequestration of HD activity from stored histones involves compartmentalization within the intact cells rather than the regulated expression of inhibitory factors. The difficulty in extracting all of the HDm from early-stage oocytes gives a pointer to possible mechanisms. Most of the HDm antigen is recovered from stage I-III oocytes only after extraction with the organic solvent Freon, which efficiently solubilizes the membranous material present in oocyte homogenates. Use of a nonionic detergent such as NP40 does not aid recovery, presumably due to loss of the membrane-associated protein into detergent micelles (extraction into the detergent phase was , in fact, achieved using Triton -X114). If not solvent extracted, the non-soluble component of HDm is lost into pellet fractions after low speed centrifugation. The presence of HDm in a non-soluble, fast-sedimenting fraction is compatible with the

immunostaining results which show the highest concentrations of reactive material at the oocyte membrane and in vesicular structures close to the cell perimeter. It is possible that these sites represent structures responsible for the anchorage of HDm in the cytoplasm. The insoluble HDm antigen has an apparent mass on SDS-PAGE of 63 kDa, which is significantly greater than that of the soluble 57 kDa form. It is likely that the higher molecular weight forms arise through modification of the HDm protein, and it is suggested here that this modification is in the form of acylation.

Although sequestration of HDm in the cytoplasm might be achieved by membrane attachment alone and would explain one way of keeping HD activity away from accumulating acetylated histones, as discussed earlier, the recovered cytoplasmic material shows little HD activity. However, HDm with associated HD activity does accumulate in the nucleus in what appears to be a fairly soluble form. Studies using chicken immature erythrocytes claim an association of nuclear HD activity with nuclear matrix material (Li et al., 1996). While we have no evidence for such an association, it is interesting to note that immunoreactivity in later stage oocytes occurs around the periphery of the nucleus and not throughout the nucleoplasm. As mentioned earlier, oocyte chromatin, organized as lampbrush chromosomes, is located internally in the nucleus and remains unstained with anti-HDm. Therefore, even in the nucleus, restraints may be placed on the ability of HDm to make contact with the chromatin and possibly also with the stored maternal histones. However, an alternative explanation is that the stored histones are protected from deacetylation by their close association with chaperones such as N1/N2 and nucleoplasmin.

The reason for accumulation of HDm in the oocyte nucleus is not altogether clear, but the nuclear envelope may serve as a final point of assembly of the active HD complex. This complex could then be distributed among the early embryonic cells, through cell divisions, along with components of the envelope. It is also possible that HDm has an additional role in deacetylating proteins other than histones in the oocyte nucleus: as yet no information is available on other types of acetylated protein in oocytes.

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**Figure legends**

**Fig. 1.** Representation of the HDm protein showing the location of the conserved region and the variable region, with the the highly-charged tail domain boxed black. Parts of the protein sequence used to raise the antibodies anti-ΔR/ΔH, anti-ΔV and anti-Cpep are shown below.

**Fig. 2.** Levels of soluble HDm protein and HD activity in oocytes at different stages of development. (A) Immunoblot, using anti-Cpep, of extracts from different stage oocytes. Each track contains the protein equivalent of two oocytes. Stages are from I to VI plus stage VI oocytes matured (VIM) by treatment with progesterone for 16 hours. The fusion protein GST-ΔV (0.1 μg) is used as a positive control at 48 kDa. (B) Immunoblot, using anti-Cpep, of nuclear (N) and cytoplasmic (C) proteins from stage III to stage VI oocytes. Each track contains the protein equivalent of ten nuclei or two cytoplasms. (C) Histone deacetylase activity assayed in extracts from oocytes (stages as in A) or embryos (C, 8-cell; B, mid-blastula; G, gastrula). Activity is expressed as dpm of $^{3}$H-acetate released from histone by extracts equivalent to a single oocyte or embryo (total column height). Also indicated is the relative amount/cell (%, black column height), which becomes disappearingly small as cell divisions increase in number.

**Fig. 3.** Immunostaining of sectioned ovary and embryo. (A) Fluorescence image of stage II oocytes stained with anti-Cpep, showing imunostaining restricted to vesicle-like structures (V) at the periphery of the cytoplasm. (B) Section shown in (A) viewed for DAPI staining. The oocytes are contained within a layer of follicle cells, the nuclei of which are stained (F). (C) Fluorescence image of a stage IV oocyte stained with anti-ΔV, showing immunostaining deposits (arrows) at the periphery of the nucleus (N). (D) as (C), but of a stage V oocyte showing more complete covering of immunostaining material (arrows) at the nuclear envelope. (E) Fluorescence image of a blastula-stage embryo stained with anti-ΔV, showing nuclear immunostaining (arrowheads). The cytoplasmic fluorescence is on the yolk platelets which may bind cytoplasmic antigen. (F) Corresponding DAPI staining, showing positions of nuclei and chromosomes. The bar represents 50 μm.

**Fig. 4.** Levels of HDm protein and HD activity extracted from oocyte preparations after treatment with the organic solvent Freon. (A) Immunoblot, using anti-Cpep, of extracts from different stage oocytes homogenized in Freon. Each track contains the protein equivalent of two oocytes. Stages as described in Fig. 2. (B) Immunoblot, using anti-Cpep, of nuclear (N) and cytoplasmic (C) proteins after Freon extraction.

Solubilized material from stage III to stage VI oocytes was analysed. Each track contains the protein equivalent of ten nuclei or two cytoplasms. (C) Histone deacetylase activity assayed in extracts from nuclei (N, black columns) and cytoplasms (C, cross-hatched columns). Activity is expressed as dpm of $^{3}$H-acetate released from histone by extracts equivalent to a single oocyte. Of the two columns in each set, the left-hand one is for homogenates treated with Freon and the right-hand one is for soluble extracts.

**Fig. 5.** Characterization of the membrane-bound 63 kDa protein. (A) Immunoblot, using anti-Cpep, showing partitioning of the 63 kDa antigen from the membrane vesicle fraction into the Triton-X114 phase (TX) rather than the supernatant (S). (B) Immunoblot, using anti-Cpep, showing proteins from the membrane vesicle fraction treated with increasing concentrations of hydroxylamine. The fusion protein GST-ΔV (0.1 μg) is used as a positive control at 48 kDa. (C) As (B), but showing a comparison of treatment with 1M hydroxylamine (HA) with treatment with 2 U/μl of alkaline phosphatase (AP). (D) Immunoblots of membrane vesicle (V) and soluble (S) fractions using anti-ΔV, anti-ΔR/ΔH and anti-CK2α.

**Fig. 6.** Characteristics of oocyte and embryo HD activity. (A) Sensitivity to trichostatin A (TSA). (B) Sensitivity to zinc ions. (C) Sensitivity to treatment with alkaline phosphatase. Soluble extracts from stage VI oocytes (black columns) were incbated in the absence (-) or presence (+) of alkaline phosphatase (2U/μl of extract).

**Fig. 7.** Sedimentation analysis of protein complexes that contain HDm protein and HD activity in developing oocytes. (A) Activity profiles of soluble extracts from previtellogenic (PV, white squares) ovary (including ~1,000 stage I oocytes) and from 25 stage V oocytes (black circles). Peak sedimentation positions of molecular mass markers (M*r*) are shown (crosses). (B) Immunoblot, using anti-Cpep, of PV material recovered from alternative fractions in (A) reacted with anti-Cpep. The fusion protein GST-ΔV (0.1 μg) is used as a positive control at 48 kDa. (C) As (B), but from a gradient used to separate a Freon-treated sample. (D) Immunoblot of stage V material recovered from alternate fractions in (A) reacted with anti-ΔV.

**Fig. 8.** Sedimentation analysis of protein complexes that contain HDm protein and HD activity in full-grown oocytes, mature oocytes and early embryos. (A) Activity profiles of soluble extracts from 50 stage VI oocytes (black circles) and from 50 stage VI oocytes that had been matured for 16 hours by treatment with progesterone (white squares). Peak sedimentation positions of molecular mass markers (M*r*) are shown (crosses). (B) Activity profile of soluble extract from 100 nuclei (black

circles) isolated from stage VI oocytes. (C) Immunoblot, using anti-Cpep, of stage VI nuclear material recovered from alternative fractions in (B). The material pelleted in the gradient (P) and the fusion protein GST-ΔV are included in the blot. (D) As (C), but from a gradient used to separate the Freon-treated extract of 100 stage VI cytoplasms. This blot is overexposed to show that the very small amount of the 57 kDa protein isolated from the cytoplasm is contained in particles sedimenting at the same rate as those containing the nuclear protein. (E) As (C), but from a gradient used to separate the Freon-treated extract of 50 blastula stage VI embryos.

**Fig. 9.** Specific immunoprecipitation of HD activity from a soluble stage VI oocyte extract. Samples eluted from anti-Cpep resin (a-P, black columns) are compared with samples eluted from control resin (C, cross-hatched columns). Unbound (UB), first wash (W1), second wash (W2), third wash (W3) and resin-bound (bound) fractions were assayed for HD activity and expressed as a percentage of the total activity applied to the resin.