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**A STUDY OF HETEROLOGOUS EXPRESSION
OF Xenopus laevis HISTONE H5**

by

PHILIP S. SHWED B. Sc. (Hons.)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE

Department of Biology, CARLETON UNIVERSITY

Ottawa, Ontario

May, 1991

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Thesis co-supervisor

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ABSTRACT

In higher eukaryotes, linker histones may mediate chromatin condensation and nuclear inactivation by contributing to structures that make DNA inaccessible to binding proteins. Study of *Xenopus laevis* H5 (XLH5) function was attempted by expression in two different organisms. Partial XLH5 cDNA, under control of a bacterial gene promoter (*lacZ*), was expressed at low level with no effect on growth of *Escherichia coli*. The expression product was identified by the use of anti-XLH5 antibody. Lack of effect may be the result of absence of heterotypic binding sites for H5 in prokaryotic chromatin. In *Saccharomyces cerevisiae*, XLH5 cDNA was engineered to be under transcriptional control of the yeast *GAL 10* promoter. One construct regulated accurate transcription and expressed an immunoreactive protein with no effect on growth. Expressed XLH5 may have been non-functional because it was smaller than authentic XLH5 in SDS-PAGE. Another construct directed constitutive transcription without XLH5 expression because of an altered *GAL 10* promoter.

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ABBREVIATIONS

2 X YT	bacterial medium (Materials and Methods section A)
2-μm circle	naturally occurring extrachromosomal <i>S. cerevisiae</i> plasmid
³²p	radioactive isotope of Phosphorus (half life: 14.3 days)
³⁵S	radioactive isotope of Sulphur (half life: 87.1 days)
A	adenine
aa	amino acid
Ap	ampicillin
Ap^r	ampicillin resistance
APS	ammonium persulphate
ATP	adenosine triphosphate
b,bp	base, base pair
BPB	bromophenol blue
BSA	bovine serum albumin
C	cytosine
C-terminus	carboxy-terminus
cDNA	complementary DNA
CEF	chicken embryo fibroblast
Ci	Curie
cpm	counts per minute
CYC-1	iso 1-cytochrome c
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate etc.
ddH₂O	distilled deionized water

ddNTP	2'3'-dideoxynucleoside 5'-triphosphate
Denhardts solution	50 X stock (0.5 L): 5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin (fraction V)
DEPC	diethylpyrocarbonate
dITP	7-deaza dGTP
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleoside 5'-triphosphate
dsDNA	double stranded DNA
DTT	dithiothreitol
EDTA	ethylene diamine tetracetic acid
EtBr	ethidium bromide
EtOH	ethanol
EUSF	erythroid cell upstream stimulatory factor
FASTA	National Research Council of Canada implementation of the Lipmann- Pearson search algorithm
G	guanine
GAL	D-galactose
<i>GAL10</i>	uridine diphosphoglucose 4-epimerase gene
h	hour(s)
H1,H5 etc.	histones H1, H5 etc.
HCl	hydrochloric acid
°C	degree Celsius
IgG(H+L)	immunoglobulin (heavy + 2 light chains)
IPTG	isopropylthiogalactoside

K	kilo
kb, kbp	kilobase, kilobase pair
kDa	kilodalton
<i>lacZ</i>	<i>β</i>-galactosidase gene
LB	Luria-Bertanini broth (see Materials and Methods A)
<i>LEU2</i>	<i>β</i>-isopropylmalate dehydrogenase
LH	linker histone
LHE	linker histone extract
μL	microliter
log	logarithm to the base 10
<i>MAT</i> (<i>a</i>, <i>1</i>, <i>2</i>)	yeast mating type locus that specifies three polypeptides that are components of regulatory proteins
MCM1	Minichromosome Maintenance 1 Yeast DNA binding protein also known as PRTF (P box recognition factor)
MgCl₂	magnesium chloride
mmol	millimolar
MNase	micrococcal nuclease
m.o.i.	multiplicity of infection
mol	Mole
M_r	relative molecular mass
mRNA	messenger RNA
MS-222	tricaine methanesulfonate
MW	Molecular weight
N-terminus	amino-terminus
NaOAc	sodium acetate

NH₄	ammonium
nm	nanometer
NRCC	National Research Council of Canada
nt	nucleotide(s)
OD	optical density
oligo (dT)	chains of thymidylic acid up to 30 nt long
ORF	open reading frame
p.i	post induction
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pfu	plaque forming units
PMSF	phenylmethylsulfonyl fluoride
pol	polymerase
poly(A +)	polyadenylated mRNA
poly(A-)	non-polyadenylated RNA
RF	replicative, double stranded form of M13
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
S	Svedberg unit
SCE	Yeast transformation solution (Materials and Methods section E2a)
SD	Shine-Delgarno site
SDS	sodium dodecyl sulphate
SED	Yeast transformation solution (Materials and Methods section E2a)

SOS	Yeast transformation solution (Materials and Methods section E2a)
SSC	0.15 M NaCl, 0.015 M Na₃citrate pH 7.6
STC	Yeast transformation solution (Materials and Methods section E2a)
ssDNA	single stranded DNA
T	thymine
TAE	40 mM Tris-acetate, 2 mM EDTA pH 8.0
TB	Terrific broth (Materials and Methods section A)
TBE	89 mM Tris-borate, 89 mM Boric acid, 2 mM EDTA pH 8.0
TBS, TBST	Tris-buffered saline with and without Tween-20 (Materials and Methods section G)
TBST	Materials and Methods section G
TCA	Trichloroacetic acid
TE	10 mM Tris-HCl, 1 mM EDTA (pH 8.0)
TEMED	tetramethylethylenediamine
tet^r	tetracycline resistance gene
Tris	2-amino-2(hydroxyl)aminomethane
tRNA	transfer RNA
TSS	bacterial transformation and storage solution Materials and Methods section E
UAS	upstream activation sequence
UPE	upstream promoter element
USF	upstream stimulatory factor
UTR	5' or 3' untranslated region of mRNA

UV	ultraviolet light
v/v	volume per volume
w/v	weight per volume
XC	xylene cyanol
XLH5	<i>Xenopus laevis</i> histone H5
YEA, YNB, YPD	yeast media (Materials and Methods section B)

I. INTRODUCTION

A. Overview

This thesis concerns the expression of *Xenopus laevis* histone H5 (XLH5), a linker histone, in both a prokaryote and a lower eukaryote. As will be detailed, histone H5 is one variant of a sub-class of a subset of nuclear proteins, known as histones (refer to table 1), that accomplish higher eukaryotic chromatin compaction. Histone H5 is found in nucleated erythrocytes in some but not all avian and amphibian species. Histone H5 accumulates during the maturation process of avian erythrocyte nuclei which, when mature, are translationally and transcriptionally inactive. XLH5 is homologous to avian H5 as defined by metabolic and molecular criteria (Rutledge *et. al.*, 1984, Rutledge *et. al.*, 1988). Although linker histones, such as H5, are important for the formation of higher order chromatin structure, how much H5 histones contribute to the differentiation of avian red blood cells is unknown.

Lower eukaryotes, such as the yeast *Saccharomyces cerevisiae*, contain a chromatin that is structurally similar to that of a higher eukaryote, but their chromatin apparently lacks a linker histone. A more extreme example of a chromatin that lacks a linker histone is the chromosome (nucleoid) of the bacterium *Escherichia coli*. Expression of XLH5 in such organisms could be used as a test of histone H5 function. In order that the reader appreciate the work reported in this thesis in the context of earlier studies, nuclear chromatin organization in higher eukaryotes, such as vertebrates, is compared and contrasted with nuclear chromatin organization in the lower eukaryote *S. cerevisiae* and the nucleoid of the prokaryote *E. coli*. The topics of contribution of linker histones to higher eukaryotic chromatin structure and inhibition of gene expression as well as evidence for the existence of histone-like proteins in *S. cerevisiae* and prokaryotes will also be introduced.

Table 1. Characteristics of five classes of *Xenopus laevis* histone proteins

Class	Classification based on amino acid composition	Molecular weight	Classification based on location	Degree of conservation
H1A	very lysine rich	21 330 [†]	linker	varies markedly between tissues and species
H1B		22 409 [†]		
H1C(1)		22 187 [*]		
H1C(2)		22 589 [*]		
H2A	lysine rich	13 932 [†]	nucleosome	moderate variation between tissues and species
H2B		14 299 [†]		
H3	arginine rich	15 352 [†]	nucleosome	highly conserved
H4		11 336 [†]		
H5A	very lysine rich	21 019 ^c	linker	
H5B	very lysine rich	20 931 ^c	linker	

[†] calculated from *Xenopus laevis* genomic sequences (Perry *et. al.*, 1985)
^{*} reported calculation from two diverged *X. laevis* genomic sequences "XLHW2" and "XLHW8" respectively (Turner *et. al.*, 1983)

^c calculated from *XLH5A,B* cDNA sequences in Rutledge *et. al.*, 1988

B. Higher eukaryotic chromatin

1. Structural features

During digestion of nuclear DNA with micrococcal nuclease (MNase) until half of the DNA is degraded to acid solubility (the so-called limit digest), several intermediates occur (reviewed in Simpson, 1978). The first of these is a 40S particle which consists of 8 nucleosomes in a compact structure. The second, about 11S in size, is enriched in the nucleosome, the DNA of which corresponds to the repeat length characteristic of a particular tissue, usually 185-200 bp. The next intermediate is the chromatosome which contains about 166-168 bp of DNA and a complete set of histones (an octamer of four core histones and a linker histone) and a core nucleosomal particle which consists of approximately 146 bp of DNA wrapped around an octamer of two of each of the core histones. In eukaryotic chromatin studied to date, the 146 bp length of DNA appears invariant (McGhee and Felsenfeld, 1980). Linker histones (LH), such as H1 and H5, associate with linker DNA (figure 1) that connects one nucleosome core to the next. The length of linker DNA varies in length up to about 80 bp.

The predominant form of chromatin in higher eukaryotic cells is a compact superhelical fibre 30 nm in diameter as detected by electron microscopy (reviewed in Felsenfeld and McGhee, 1980). How polynucleosome strings are folded to form this fibre is not known. The eukaryotic chromatin fibre is a dynamic structure. EM spreads of chromatin transcribed by RNA polymerase II (RNA pol II) show a high concentration of nucleosomes associated with genes during low transcriptional activity (De Bernardin *et. al.*, 1986) and apparent absence on genes with high transcriptional activity such as in a secretory protein gene in the salivary glands of *Chironomus* (Widmer *et. al.*, 1984). Biochemical data support the occurrence of change in conformation in transcriptionally active fibres. Highly transcriptionally active genes can be differentiated on the basis of micrococcal nuclease (MNase)

Figure 1. Structural features of eukaryotic chromatin

cleavage patterns. MNase cleavage of transcriptionally active DNA generates a multimeric ladder (Bellard *et. al.*, 1979) which becomes progressively blurred with increasing gene activity (Wu *et. al.*, 1979).

Linker histones (LH) have long been believed to mediate chromatin compaction, since removal of LH from condensed chromatin caused it to dissociate into a diffuse network of fibrils and restoration of LH produced clumps of condensed chromatin (Littau *et. al.*, 1965). Changes in morphology of native and H1 depleted chromatin have also been studied at different ionic strengths (Thoma *et. al.*, 1979). H1 containing chromatin at low ionic strength (10 mM NaCl, pH 7.0) was observed to possess fibers with clearly visible nucleosomal beads. With increasing ionic strength, nucleosomes became more compact and stable. In contrast, removal of H1 resulted in a dispersed chromatin structure that condensed, with increasing ionic strength, into clumps that lacked fiber morphology.

Histones H1 and H5 were dissociated from chicken erythrocyte chromatin and replaced with purified H1, H5 and calf thymus H1 in order to determine the properties of each type of chromatin (Allan *et. al.*, 1981). These studies established that higher order chromatin structure depended on linker histone interactions and, as the linker histone to DNA ratio increased from one to two, a 50% increase in packing ratio was observed.

2. Linker histones

a) Linker histone classification

LHs are the most variable of the five classes of histones and show tissue and species specificity (Cary *et. al.*, 1981). Two subclasses of LH, referred to here as H1 and H5, appear to exist according to the criteria of coupling of linker histone synthesis to DNA replication and of sequence relatedness.

(1) H1 subclass

Most vertebrate cells contain H1 histones as their major LH in the form of

half a dozen subtypes (Cole, 1984). This subclass possesses several metabolic and structural characteristics similar to those of core histones: synthesis coupled to DNA replication (Borun, 1975; Pederson, 1976; Maxson *et. al.*, 1983), synthesis from nonpolyadenylated mRNA (Kedes, 1979; Hentschel and Birnstein, 1981) with conserved dyad symmetrical 3' UTR sequences (Doenecke and Tonjes, 1986), and linkage to core histone genes within known gene clusters (Kedes, 1979; Hentschel and Bernstein, 1981; Maxson *et. al.*, 1983).

(2) H5/H1° subclass

This subclass of LH is composed of two major families: mammalian H1° and non-mammalian H5. Mammalian H1° is found in several differentiated tissues (Panyim and Chalkley, 1969) and possesses metabolic and molecular similarities with H5. H1° synthesis occurs independent of DNA replication (Chabanas *et. al.*, 1983; D'Anna *et. al.*, 1985) and increases in amount during reduced proliferative activity in many cultured mammalian cells. Even though mammalian H1° and avian H5 share extensive amino acid sequence homology, they probably function differently based on broader tissue distribution of H1° and higher arginine content in H5 (Doenecke and Tonjes, 1986).

Histone H5 constitutes a significant fraction of nuclear protein from mature erythrocytes of some avian and amphibian species (Neelin *et. al.*, 1964; Hnilica, 1964; Destree *et. al.*, 1979; Rutledge *et. al.*, 1984). During erythrocyte maturation, nuclei become condensed (Kernall *et. al.*, 1971; Brasch *et. al.*, 1971; Maclean *et. al.*, 1974) and the cells become translationally and transcriptionally inactive (Billet and Hindley, 1972; Seligy *et. al.*, 1973). H5 accumulates during the erythrocyte maturation process and for this reason has been suggested as a chromatin repressor (Seligy and Neelin, 1970; Hannon *et. al.* 1984; Schlissel and Brown, 1984; Weintraub, 1984; reviewed in Weintraub, 1985; Clark *et. al.*, 1988).

Synthesis of avian H5 is not coupled with DNA replication, as shown by insensitivity of synthesis to cytosine arabinoside (Seligy *et. al.*, 1973), and persists into the final stages of erythrocyte maturation (Appels and Wells, 1972; Seligy *et. al.*, 1973; Ruiz-Carrillo *et. al.*, 1976, 1983; Sung *et. al.*, 1977). However, transcription of the chicken H5 gene occurs throughout the cell cycle of a lineage of chicken erythroid cells (Dalton *et. al.*, 1986). H5 can also be distinguished from H1 by molecular features such as polyadenylation of its mRNA (Molgaard *et. al.*, 1980; Rutledge *et. al.*, 1988), which lacks the conserved dyad sequence present in H1 and core mRNA (Doenecke and Tonjes, 1984; Coles and Wells, 1985; Rutledge *et. al.*, 1988), and by absence of linkage to core histone genes (Krieg *et. al.*, 1983; Ruiz-Carrillo, 1984).

(b) Xenopus laevis histone H5

Since this thesis concerns XLH5, it is appropriate to review literature pertinent to this specific LH, as well as determination of function of H5 histones and regulation of H5 gene expression in general. XLH5 is found in the erythrocytes of the South African clawed toad *Xenopus laevis* and in addition H5-like histones are found in several tissues, with the exception of early embryonic stages and mature sperm and eggs (Moorman *et. al.*, 1986). Two proteins that may be closely related to XLH5 were found to be differentially expressed in several adult tissues of *X. laevis* such as testes, livers and erythrocytes (Risley and Eckhardt, 1981; Brown *et. al.*, 1981). None of these proteins was proven to be an H5 histone although various evidence suggests this identity. Electrophoretically, the proteins were indistinguishable from XLH5 and following *Staphylococcus aureus* V8 proteinase treatment, a similar pattern of peptides was seen in SDS gels (Risely and Ekhart, 1981). Also, chromatographic properties of PCA-extracts from nuclei of *X. laevis* erythrocytes and livers were the same and amino acid compositions were similar (Brown *et. al.*, 1981).

As already mentioned, XLH5 has metabolic characteristics similar to avian H5 (Rutledge *et. al.*, 1984) and this suggests that both function similarly. XLH5 synthesis appears to be independent of DNA replication during late stages of erythrocyte maturation to the extent of three fold accumulation relative to H1. Although *Xenopus* and chicken red blood cells both contain a total amount of LH of about 28% of all histone fractions, the latter have only 9% H5 (Destree *et. al.*, 1979), whereas chicken erythrocytes have up to 23% H5 (Panyim *et. al.*, 1971). The reason for this difference is still not known.

Definite proof of an avian H5-like histone in *X. laevis* red blood cells was provided by Rutledge *et. al.* (1988) with the identification of two XLH5 cDNA clones which may correspond to the two putative H5 variants H1D and H1E, described by Risley and Eckhardt (1981) and also reported by Brown *et. al.* (1981). Evidence that the cDNAs code for H5-like lysine rich variants came from sequence alignment with an octapeptide from the major XLH5 erythrocyte protein (Brown, 1982), extensive nucleotide and amino acid sequence similarity with both avian H5 and human H1^o sequences and low degree of sequence similarity to known eukaryotic H1 genes (Rutledge *et. al.*, 1987).

Characterization of XLH5 mRNA by cDNA sequence analysis (Rutledge, 1987; Rutledge *et. al.*, 1988) revealed resemblance to mRNA of both avian H5 (Molgaard *et. al.*, 1980) and human H1^o (Kress *et. al.*, 1986) with respect to polyadenylation and lack of a dyad symmetrical element in the 3' UTR. These observations complement the metabolic studies that distinguish this subclass of linker histone from H1 (Rutledge *et. al.*, 1984). Primer extension analysis (Foster, 1990) and sequence analysis (Rutledge *et. al.*, 1988) further revealed that XLH5 mRNA is distinct from avian H5 and human H1^o in featuring a large 1.25 kb 5' UTR. In contrast, avian H5 and human H1 mRNA possess short 5' UTR (Ruiz-Carrillo *et. al.*, 1983; Doenecke and Tonjes, 1984; Kress *et. al.*, 1986). The long UTR

may function in stabilizing XLH5 mRNA (Foster, 1990). However, the 3' UTR of *XLH5* is comparable to the average observed for avian H5 (approx. 200 bp) (Rutledge *et. al.*, 1988; tabulated in Foster, 1990) but shorter than that of mammalian H1^o. Northern blot analysis revealed that H5 messages from different erythroid cell types were of the same length, and shortening of either the 5' or 3' end including the poly(A) tail was not evident as the cells matured (Foster, 1990). *In vitro* transcription of H5 mRNA, isolated from nuclei of erythrocytes, could only be detected from early stages of recovery from anemia. This suggests that either only a small amount of XLH5 is transcribed and the message is very stable or transcription occurs only at early stages of development.

The relationship of *XLH5* to known gene clusters (Perry *et. al.*, 1985) has not been determined. As in a typical higher eukaryote, histone genes of *X. laevis* are arranged in several dispersed clusters, each usually containing one or more genes for the five histone classes, including H1 (reviewed in Maxson, 1983). For comparison, the chicken H5 gene is unlinked to clustered core and H1 genes in the chicken genome (Krieg *et. al.*, 1983).

(c) Function of linker histones

The inverse correlation between linker histone content and chromatin condensation has been documented since the mid 1960's and it has long been believed that linker histones such as H1 or one of its variants may keep chromatin inactive.

Several studies carried out during the last decade illustrate the inverse relationship between histone H1 and transcriptional activity. H1 appears to bind to transcriptionally inactive and active regions of chromatin in different ways. In the former case, adjacent nucleosomes are cross-linked and in the latter case, binding appears to occur in a way that does not hold the nucleosomes together (Weintraub, 1984). In a second study, *in vitro* transcription of transcriptionally inactive genes, in

somatic chromatin, took place when H1 was removed (Schlissel and Brown, 1984). More recently, it was demonstrated that addition of H1 to an extract of *X. laevis* oocyte nuclei, that was deficient in H1, resulted in dominant and selective repression of class III genes, as exemplified by oocyte 5S RNA and satellite I DNA (Wolffe, 1989). Also, an entirely different function has been proposed for LH in the recent demonstration that H1 is a recombinase that catalyzes ATP-independent DNA strand transfer (Kawasaki *et. al.*, 1989). A crude extract of protein from calf thymus was able to catalyze strand transfer from a linear double stranded tetracycline resistance (tet^r) gene to circular tet^r in the bacteriophage M13mp8. The amino acid sequences responsible for this putative strand transfer activity are unknown.

As H5 accumulates in the avian erythrocyte, there is a 70% net increase in linker histone content (Moss *et. al.*, 1973; Appels and Wells, 1972; Billet and Hindley, 1972) and a 12% increase in nucleosomal DNA repeat length (Weintraub, 1978). Accumulation of H5 is associated with H1 displacement (Billet and Hindley, 1972; Ruiz-Carrillo *et. al.*, 1984) or replacement (Urban *et. al.*, 1980). However, it is not known how much H5 contributes to differentiation of red blood cells and their loss of self-renewal capacity or apoptosis.

Removal of H5 has been shown to increase chromatin decondensation, the template capacity, the primary binding of intercalative dyes ethidium bromide (EtBr) and actinomycin D, as well as to decrease thermal denaturation (Seligy and Neelin, 1970; Williams *et. al.*, 1972; Seligy and Miyagi, 1974; Lurquin and Seligy, 1975). The above data together with chromatin decomposition and reconstitution studies suggest that H5 may inactivate the erythroid genome by keeping it in a pseudo-pycnotic state. Several *in vivo* studies support this inference. Avian H5 transfer to quail and rat nuclei of heterokaryons formed by fusion of myoblasts with chicken erythrocytes was believed to account for suppression of replication

(Ringertz *et. al.*, 1985). Also, rat myoblast chromatin compaction and cellular distortion were observed after microinjection of purified avian H5 into proliferating cells. Pulse labelling experiments employing radioactive precursors indicated blockage of both transcription and replication (Bergman *et. al.*, 1988).

(d) Regulation of avian H5 gene expression

In recent years attention has been placed on understanding the molecular mechanisms of avian H5 gene regulation that dictate selective expression and regulation independent of other histones, at the transcriptional and post-transcriptional levels.

In higher eukaryotes the sequence elements that are needed for gene regulation are designated promoters and enhancers (Maniatis *et. al.*, 1987). Promoters function to accurately and efficiently initiate transcription. Usually they are located upstream of the transcription initiation site and are variable in length. Several sequence elements, that bind to non-histone proteins, compose higher eukaryotic promoters: an AT-rich TATA (or Goldberg-Hogness) box and several upstream promoter elements (UPEs). The TATA box ensures that transcription takes place accurately and efficiently and UPEs increase transcription rate. Enhancers are similar to promoters in that they also contain sequence motifs that are capable of binding to proteins. Enhancers differ from promoters in being able to act on cis-linked promoters at large distances either upstream or downstream from promoters and the structural gene and also in either a forward or a reverse orientation.

Various approaches have been used to delineate sequence elements in flanking 5' and 3' regions that are involved in modulation of H5 genes. Chromatin structure of the chicken H5 locus was examined at different stages of maturation and it was observed that hypersensitivity of segments of the locus to DNase I were modulated during erythrocyte maturation in parallel with transcriptional activi., of

the gene (Renaud and Ruiz-Carrillo, 1986). The most DNase I sensitive regions were upstream of the transcription initiation site, between -1200 and -900, and in the first 1/3 of the coding region. The least sensitive regions were located upstream of the promoter and in the last 2/3 of the coding region. An erythroid-specific enhancer sequence, identified downstream of the chicken H5 coding region (Trainor *et. al.*, 1987), may correspond to a DNase I hypersensitive site in the 3' UTR (Renaud and Ruiz-Carrillo, 1986). The element was detected in the avian H5 gene using a transient transfection assay in an erythroid cell line. Definitive identification involved use of constructs in which a reporter gene (bacterial chloramphenicol acetyltransferase) was transcriptionally directed by the H5 promoter with and without the H5 3' UTR fragment.

More recently it has been suggested that the chicken H5 3' UTR, position +1042 to +1185, contains an activating erythroid-specific element with modest activity (Rousseau *et. al.*, 1989). This was determined by plasmid cotransfection into erythroid and non-erythroid cells of chicken bone marrow cells.

A 398 bp region of chicken H5, between -85 to +313, appears to contain an enhancer-like element responsive to chromatin salt wash fractions of a chicken erythroid cell line (Wigley and Wells, 1987). These transcriptional studies used *Xenopus* oocytes that were coinjected with fractions and plasmids containing the H5 gene. More recent analyses by plasmid co-transfection in erythroid and non-erythroid cells were suggestive of a tissue-specific enhancer element located within the 5' UTR at -312 to -47 of chicken H5 (Trainor and Engel, 1989).

The strategy of deletion of the chicken H5 promoter region and fusion to a reporter gene revealed a negative element (-115 to -95) which is believed to be useful in downregulating H5 transcription (Rousseau *et. al.*, 1989). Also, two positive elements (-83 to -74) and (-54 to -38) were revealed. The latter, designated 'upstream proximal element', is believed to be a specific element of the H5/H1°

subclass as it occurs in a region of homology between chicken H5, human H1^o and duck H5 and does not appear to be present in vertebrate H1 genes.

A sequence motif with homology to the consensus sequence, upstream stimulatory factor (USF) binding site of the Adenovirus 2 major late promoter (-63 to -52) was identified, between +520 and +500 in the duck H5 coding region, and designated erythroid cell USF (EUSF) (During *et. al.*, 1990). The EUSF appears to be a conserved element in avian H5 (chicken, +434 to +445; duck, +505 to +516). That EUSF was involved in transactivation of H5 was supported by electrophoretic mobility shift analyses and footprint assays with DNase I. Deletion of a fragment containing the sequence abolished the transactivation which EUSF exerted in a cell free system.

e) Linker histone protein structure and binding

Linker histones H1 and H5 are proteins with unusual amino acid compositions, that are believed to have a multi-domain structure consisting of an N-terminal domain of variable length (13-40 amino acid (aa) residues), a central trypsin resistant globular domain (80 aa residues) and a long C-terminal tail (85-100 residues) (reviewed in van Holde, 1989).

The biological function of the N-terminal domain is not known. The N-terminal domains of both H1 and H5 are lysine-rich; however, the N-terminal domain of histone H5 is smaller than that of H1 (in avian H5 it is approximately 30 residues, whereas in rabbit or calf H1 it is approximately 40 residues). Similar stretches of amino acids occur within both H1 and H5 (Seligy *et. al.*, 1976; Yaguchi *et. al.*, 1977).

The globular domains of linker histones are believed to seal nucleosomal DNA. When peptide fragments of H1 were added to H1-depleted chromatin, and the product digested with MNase, only the fragments containing the globular domain resulted in a 166 bp chromatosomal pause (Allan *et. al.*, 1981). Superficially,

the globular domains of H1 and H5 are similar. Most of the folded structure of H5 and H1 is found between residues 22 and 100. The conformations of the globular domains of H1 and H5 are believed to be different, as observed in spectroscopic and NMR studies by Cary *et. al.* (1981) and by the fact that antibodies against the globular region of H5 would cross-react with only H1^o but not H1 (Allan *et. al.*, 1984).

The C-terminal domain of H1 and variants is believed to be responsible for neutralizing the charge of the phosphate backbone and appears to be required for chromatin condensation (Allan *et. al.*, 1986). This domain of an LH protein is very basic; up to half of the amino acids are lysines and arginines. Histone H5 is proposed to have a higher binding affinity than H1 in erythrocyte nuclei (Kumar and Walker, 1980) because of the presence of more arginine in the H5 tail than in H1 (13 of the 22 arginines in H5 are contained in the 89 residue carboxy-terminal tail).

C. Yeast chromatin

The unicellular eukaryote *S. cerevisiae* is a recent experimental organism for the study of histones (Smith, 1984). Since the chromatin structure of yeast genes has been comprehensively reviewed (Perez-Ortin *et. al.*, 1989) the following discussion underscores features believed to be related to absence of an H1 histone.

Yeast chromatin has a nucleosomal structure (Lohr and Van Holde, 1975). As in most eukaryotes, 146 bp of DNA are associated with the core histone complex. The observed repeat length of yeast chromatin is 160-165 bp, which corresponds to a linker DNA length of 20 bp (Lohr and Van Holde, 1975; Nelson *et. al.*, 1977; Thomas and Furber, 1976). The repeat length is relatively short as compared to other metazoan eukaryotes (eg. *X. laevis* erythrocyte DNA repeat length: 184 ± 4 (Gottesfield, 1980)). Nucleosomal organization also occurs in *S. cerevisiae* nuclear plasmids, such as 2- μ m which has a repeat length of approximately 168 bp

(Livingston and Hahne, 1979; Nelson and Fangman, 1979; Seligy *et. al.*, 1980).

At mid-logarithmic growth, transcribed yeast chromatin and total chromatin are equally sensitive to DNase I (Lohr and Hereford, 1979), a finding which contrasts with similar DNase I experiments using higher eukaryotic genes such as the chicken globin gene, which has a conformation that varies with tissue (Weintraub and Groudine, 1976). However, yeast chromatin of both genomic and 2- μ m plasmid DNA likely undergoes a conformational change at late-log growth as shown by the decreased DNase I and MNase sensitivity (Seligy *et. al.*, 1980). These observations have been interpreted as showing that the entire yeast genome is in the same conformational (metabolic) state. This may represent the same conformation which appears to exist in a small part of total chromatin of higher eukaryotes.

A high proportion of the yeast genome is transcribed, as shown in the complementary nature of 20% of single copy DNA to mRNA (Hereford and Rosbash, 1977). Given that the genome has been estimated to code for approximately 5- 10,000 mRNA sequences, the above result indicates that at least 40% of the yeast genome is transcribed and contrasts with the relatively low value in higher eukaryotes, where transcriptionally active sequences of less than 5% of the total genome are more usual. Interestingly, the size of the transcriptionally active proportion of the yeast genome is comparable to that of a higher eukaryotic genome.

However, another feature of yeast chromatin that was believed to be related to linker histone absence has now been shown to be false. During the 1970's, mitosis in *S. cerevisiae* received interest because the changes in chromatin topology that characterize mitosis in higher eukaryotes were not observed. Chromatin appeared neither to condense into chromatids during nuclear division nor to aggregate into metaphase or anaphase plates. The appearance of metaphase chromatin was deemed to be the same as in interphase (Gordon, 1977). These observations shaped

perception of the folded state of yeast chromatin for the next decade, until computer enhancement of images of yeast nuclei obtained in 1987 by Hiraoka demonstrated the presence of chromosome-like rods with a diameter of 28 nm.

1. Yeast Histones

a) Core

Yeast DNA is associated with the four nucleosomal inner histones: H2A, H2B, H3 and H4 (Wintersberger, 1973), that are similar to and capable of interaction with their mammalian counterparts as shown in the interkingdom complexes between yeast and calf H2A and H2B (Mardian and Isenberg, 1978).

b) Gene structure

The haploid genome of *S. cerevisiae* contains two copies of each of the core histone genes, all of which are transcribed and translated. The genes are arranged as two nonallelic sets of H2A-H2B gene pairs and two nonallelic sets of H3-H4 pairs. At all loci, the genes are arranged head to head and are divergently transcribed from a 600-800 bp intergenic region (Hereford *et. al.*, 1979; Smith and Murray, 1983; Smith, 1984).

One hypothesis that accounts for the observed non-allelic gene pair duplication involves fusion of two primitive fungal genomes, each of which possesses one set of core histones, to give rise to a defective diploid genome that went on to evolve haploid *S. cerevisiae* (Smith, 1987). Consistent with this model is the observation of a large number of duplicated genes in this yeast.

c) Molecular Genetics of Yeast Histones

Manipulation of *S. cerevisiae* core histone genes has revealed abundant information on the function of individual histones and/or their domains in chromatin structure. This research has involved use of two kinds of experiments. In one approach, one of the genes for a given histone was inactivated by replacement with a homologous gene containing a frameshift mutation. This allowed further

manipulation of the remaining active gene by *in vitro* mutagenesis. Alternatively, both of the genes for a given histone class were inactivated and an extrachromosomal engineered version of one of them was introduced by transformation using a plasmid.

Such strategies have allowed the demonstration of functional redundancy of duplicated gene pairs. Yeast can survive with either of the two H2B (Rykowski *et al.*, 1981) or H2A subtypes alone (Kolodrubetz *et al.*, 1982).

Deletion of the N-terminal regions of H2B (amino acids 3-22, 14-31) or of the variant H2A.1 (not to be confused with H4) also had no consequence for yeast growth, but a deletion removing amino acids 13-70 was lethal (Wallis *et al.*, 1983; Schuster *et al.*, 1986). Deletion of the N-terminal tail of H4 (amino acids 4-22, 4-19, 4-23, 4-28) was also observed to be inconsequential for growth, although it was necessary for the specific repression of the mating loci (α and a) (Kayne *et al.*, 1988). The deletions were associated with disruption of chromatin structure and lengthening of the yeast cell cycle, especially the G2 phase. Any combination of H2A subtypes or of H2B subtypes produced viable cells (Kolodrubetz *et al.*, 1982). Chimerical histones produced by exchange of N-terminal tails between H2A and H2B are completely functional (Schuster *et al.*, 1986). Yeast strains have been constructed in which a single functional gene for H2B (Han *et al.*, 1987) or H4 (Kim *et al.*, 1988) was placed under the control of the *S. cerevisiae* GAL1-10 promoter which allowed specific induction of histone gene expression in galactose and repression by glucose (reviewed in Johnston, 1987). This strategy has allowed demonstration of the necessity of H2B and H4 for growth and the consequent cell cycle arrest resulting from removal of either histone.

Maintenance of correct histone stoichiometry appears to be required *in vivo*. When histone gene dosage in *S. cerevisiae* was increased or decreased, effects were seen on processes such as transcription, chromosome replication, sporulation and

germination (Osley and Moran, 1988).

d) Status of linker histone existence in yeast

Features of *S. cerevisiae* total chromatin such as similarity in DNase I sensitivity to that of transcriptionally active genes of a higher eukaryote (Lohr and Hereford, 1979) and relatively short DNA repeat length, are suggestive of H1 absence. At least one immunoblot analysis, that used an antibody for the globular domain of H5/H1, did not identify any H1-like histones in a perchloric acid extract of *S. cerevisiae* chromatin (Smith *et. al.*, 1984).

The presence of an H1-like histone in distantly related *Schizosaccharomyces pombe* is also uncertain (Matsumoto and Yanagida, 1985). Although a probe for *Drosophila* H1 hybridized with several clones of a genomic library from *S. pombe*, the nucleotide sequences of restriction fragments from these clones did not reveal a reading frame corresponding to H1.

Use of MNase on baker's yeast chromatin not only released core histones, but also a protein of 20 kDa size with amino acid composition expected for an H1-like protein (Certa *et. al.*, 1984). However, with immunological work and cell fractionation, it was established that the protein was the mitochondrial HM protein. Previous characterization of this abundant yeast mitochondrial protein had revealed that it featured histone-like characteristics such as being rich in lysine, basic as well as properties such as being heat-stable and able to introduce superhelical turns into circular DNA (Caron *et. al.*, 1979).

Immunoblotting analysis of a protein-rich salt/perchloric acid (PCA) extract of *S. cerevisiae* proteins with polyclonal antiserum to mouse liver H1 revealed three reactive fractions (Srebrevia *et. al.*, 1987). Two of the fractions had electrophoretic mobilities close to calf thymus H1 and, although sizes of proteins were not indicated, they are believed to represent H1 subtypes. The third fraction of higher molecular mass was believed to be a dimer. One criticism that can be made of this

study is that precise specificity of the antibody was not shown by demonstrating which LH determinants react with the antibody, but this antiserum also reacts with PCA-soluble cytoplasmic proteins from mouse-liver and Friend erythroleukemia cells (Zlatanova *et. al.*, 1990).

Although yeast may not have an H1-like histone, it may have one or more proteins that serve the role of mediation of higher order chromatin compaction. Dynamic light scattering, electron microscopy and X-ray diffraction experiments show that yeast chromatin undergoes a cation-dependent folding into 30 nm filaments that resemble those of higher eukaryotic chromatin. Packing of nucleosomes within the 30 nm filaments is similar to those of higher cells (Lowary and Widom, 1989).

D. Bacterial chromatin

Prokaryotic chromatin organization is considerably different from that of a eukaryote. Under the electron microscope the nucleoid (bacterial chromosome) appears as a discrete compact structure that is lobular in shape with clefts (Hobot *et. al.*, 1985; reviewed in Schmid, 1988; Drlica 1987a,b) (figure 2). It is not known whether the nucleoid is a dynamic or static structure, although inhibition of bacterial protein synthesis results in morphological change (Bjornsti *et. al.*, 1986). Metabolically active regions of the nucleoid occur on the edges as shown by presence of single stranded (ss) nucleic acids, nascent RNA and ribosomes. It has been speculated that transcriptionally active genes are positioned at the edges of the nucleoid and when translation is blocked, the genes are returned to the condensed structure.

A few aspects of chromatin organization are similar in prokaryotes and eukaryotes. Electron microscopy of partially lysed *E. coli* revealed a potential prokaryotic equivalent of the nucleosome in observed labile 12 nm filaments with an axial repeat of 13 nm (Griffith, 1976). Another comparable feature is the fact that

Figure 2. Structural features of the bacterial nucleoid

the bacterial nucleoid appears to be organized into domains that are comparable in size to the 50 -100 kbp domain size of eukaryotic chromosomes (Sinden and chromatin. The most pertinent of these to this study is that an intermediate level of Pettijohn, 1981). Significant differences occur between prokaryotic and eukaryotic chromatin compaction, similar to that when higher eukaryotic chromatin binds to H1, is absent in prokaryotes (reviewed in Schmid, 1988). Also, most eukaryotic DNA is not torsionally strained as are the negatively supercoiled domains of the nucleoid. Finally, eukaryotic nucleosomes are organized in a repeating structure with multimers of nucleosome-bound DNA of discrete size, whereas the prokaryotic nucleoid has no analogous organization.

1. Prokaryotic Histone-like proteins

A clear definition of bacterial chromatin does not exist. The nucleoid can be isolated as a DNA-RNA protein complex when cells are lysed in the presence of sufficient counterions to stabilize DNA charge-charge repulsion. Depending on the counterion used, the associated macromolecules differ significantly (reviewed in Schmid, 1988). Nucleoids isolated using spermidine as a counterion may include attached membrane as well as associated proteins which include 'histone-like' proteins of basic or neutral charge and the capacity to bind to DNA. Bacterial histone-like proteins have been reviewed recently (Drlica, 1987a,b; Schmid, 1988) and their characteristics are summarized in Table 2.

Proteins HU and H1 were believed to be significant in organization of bacterial chromatin structure (Drlica, 1987). These proteins constitute a large portion of the protein associated with DNA, and each of the two is calculated to associate with 150-200 bp DNA. Micrococcal nuclease digestion of nucleoid fragments results in production of two small DNA-protein particles, of 160 and 120 bp each. Based on immunocytochemistry data, HU does not have a role in the

Table 2. Prokaryotic histone-like proteins ^a

Protein	Molecular weight	Net Charge	Copies/Cell	Histone Features
HU	9000	basic	60 000	
HLP1	17000	basic	20 000	
H	28000	basic	120 000	Recognized by anti-H2A antibody
H1a,b,c	16000	neutral	20 000	Strong DNA binding. Affects in vitro transcription.

^a revised from Schmid, 1988

formation of nucleosomal structures (Durrenberger *et. al.*, 1988). HU appears to be located on the periphery of the nucleoid over ribosomes in a 60 nm wide area. The fact that DNA dependent RNA polymerase (pol) and ssDNA are located over ribosomes in the same area support the idea that HU may be involved in the transcription translation process.

Protein H is a DNA binding protein that reacted with antiserum specific for calf thymus histone H2A (Hubscher *et. al.*, 1980) and it is also identical to the *E. coli* ribosomal protein S3, based on co-migration in PAGE gels, heat stability, amino acid analysis, effects on FLP-promoted recombination in yeast (Bruckner and Cox, 1989).

Recent data suggest that *E. coli* HLP-I is an outer membrane protein (Hirvas *et. al.*, 1990). *S. typhimurium* has a 16 kDa outer membrane protein designated OmpH that is 91% homologous to HLP-I. The *E. coli* *hlpA* ORF resembles the *ompH* gene. This study does not rule out the possibility that OmpH/HLP-I are both outer membrane and DNA binding nucleoid proteins.

This description of bacterial histone-like proteins reflects how the current state of knowledge of these proteins is incomplete, especially with regard to interactions of these proteins with each other, polyamines and DNA.

E. Expression of eukaryotic proteins in *E. coli* or *S. cerevisiae*

Since the goal of this thesis was to express XLH5 in a prokaryote and a eukaryote, it is appropriate to devote a portion of this introduction to the topic of recombinant vector systems for protein expression. A number of recombinant vector systems have been developed and used to express eukaryotic proteins in both *E. coli* or *S. cerevisiae* in order to produce large quantities of proteins, that are of limited natural availability, for biochemical analysis or therapeutic use (Marston, 1987; Carter *et. al.*, 1987). High level expression of an open reading frame may enable

determination of its identity, but there are still serious problems associated with obtaining efficient high-level expression.

One of the basic requirements of expression of a protein in *E. coli* is the need for a strong, regulated promoter such as that of *trp* or *lac* (Marston, 1987). Also, in order that initiation of translation take place efficiently, it is necessary that *E. coli* ribosomes recognize a binding site that includes an upstream sequence to a Shine-Delgarno (SD) site and a spacer region between the SD site and the initiation codon (Shine and Delgarno, 1974; Curry and Tomisch, 1988).

Vector systems have been developed for intracellular or extracellular expression. Intracellular expression of proteins in *E. coli* can result in the accumulation of up to 25% of total cell protein, although most of the product may be in an insoluble and aggregated form (Marston, 1986). Products are recovered with reduced biological activity because purification techniques are required. Secretion of proteins reduces the problem of protein purification, but expressed yields of extracellular proteins are quite low.

Prokaryotes are not always very ideal for the expression of eukaryotic genes. Higher eukaryotes and *E. coli* have differing codon biases (Bennetzen and Hall, 1982), and this could hamper successful expression of a protein. Also, bacteria cannot perform intron processing and post-translational modifications, which are needed for biological activity of a protein.

Use of a yeast such as *S. cerevisiae*, as host for expression of a higher eukaryotic gene, alleviates some of the problems inherent with a prokaryote. *S. cerevisiae* is an easily cultivated, free living yeast that has been well studied with both classical and molecular genetics methods (Botstein and Fink, 1988). Like a prokaryotic microbe, the doubling time of the lower eukaryote is rapid, about twice as fast as that of *E. coli*. On the other hand, cell biology and metabolic properties of yeast are similar to those of a multicellular eukaryote. The yeast secretory pathway

has been partially detailed and is similar to that of higher eukaryotic cells. As such, yeast cells are capable of post-translational modifications such as glycosylation (addition of N-linked and O-linked carbohydrate side chains), acetylation, proper disulphide bond formation and folding. However, *S. cerevisiae* is unable to perform some types of intron processing and post-translational modifications (Van Brunt, 1986).

A number of yeast vectors have been developed which incorporate strong regulatable promoters such as glycolytic ones (Carter *et. al.*, 1987). For instance, YEp51, which was chosen to direct transcription of XLH5 in this study, contains the promoter of the *GAL10* gene (Broach *et. al.*, 1983). In *S. cerevisiae*, the *GAL10* protein (uridine diphosphoglucose- 4 epimerase), which functions in the pathway of galactose utilization (appendix 1), is highly expressed only when cells are grown on galactose to the extent that transcripts comprise between 0.25 to 1% of total polyadenylated mRNA (St. John and Davis, 1981). The *GAL10* gene is unexpressed when cells are grown in glucose (refer to discussion). In addition to promoters, yeast expression vectors incorporate separate yeast and bacterial replicons so that the vectors can be propagated in both yeast and *E. coli*. This incorporation is needed because manipulation and large scale preparation of vectors is easier and faster to carry out in *E. coli* than in *S. cerevisiae*.

F. Thesis objectives

A variety of *in vitro* and *in vivo* cytological and biochemical studies, reviewed in previous sections of the introduction, have alluded to the possible function of H5. These studies demonstrate a correlation between H5 expression and chromatin condensation as well as loss of replicational and transcriptional activity. A model consistent with these correlations involves H5 binding to chromatin and generation of a higher order structure that is inaccessible to other DNA binding proteins. This thesis is an attempt to test the above model through the

approach of heterologous expression of XLH5 in both a prokaryote and lower eukaryote which do not normally produce an authentic linker histone.

A study of expression of H5 in *E. coli* might determine whether DNA compaction mechanisms have been evolutionarily conserved. It is conceivable that interaction of a basic eukaryotic protein such as XLH5 with the bacterial nucleoid would lead to novel conformational constraints. These constraints might further result in defective replication and/or transcription and translation that could be detected by transient and persistent losses in cell growth and plasmid production (copy number). Fortunately, the groundwork for an *E. coli* expression study was already provided by the initial identification of partial XLH5 cDNA, fused to the *lacZ* gene, through immunological screening with anti-XLH5 antibody (Rutledge, 1987; Rutledge *et. al.*, 1988). What remained was to assess the physiological implications of expression of these partial H5 products and full length XLH5. Therefore, the first objectives of this thesis were to monitor growth before and after host cell induction of XLH5 expression and to analyze extracted proteins for H5 products by immunoblotting.

Since *E. coli* chromatin may not be very suitable for studying H5 function, the second and major objective of this thesis was to express XLH5 cDNA in *S. cerevisiae*. Since chromatin of baker's yeast appears to have a nucleosomal structure that is similar to that of a higher eukaryote, it may possess H1-like binding sites even though features, such as short repeat length and relative insensitivity to DNase I of transcribed regions compared with non-transcribed regions, are consistent with the absence of an LH histone. Therefore, XLH5 expression in yeast could have a range of effects on yeast physiology, such as promoting changes in chromatin compaction, loss of cell proliferation and even different morphology. To circumvent the possibility that H5 expression might be lethal to yeast, part of the research was devoted to engineering an XLH5 cDNA so that it would be expressed

in a growth-dependent manner. To this end, the inducible *GAL10* promoter was chosen for directing the expression of *XLH5* (refer to section I g). Additional subgoals of this second objective also included the engineering of the *XLH5* coding region to be in phase with the *GAL10* promoter, introduction of *XLH5* into yeast by transformation, time point northern analysis of *H5* mRNA expression in induced and non-induced transformed cells, and immunoblot analysis of *H5* products in protein extracts of yeast transformants.

II. MATERIALS AND METHODS

A. Bacterial Strains: Growth and Storage conditions

Escherichia coli JM103 was used as a host for chimeric phage containing XLH5 cDNA sequences as well as various cloned DNAs (see table 4). This strain was also used as a recipient for plasmids recovered from *Saccharomyces cerevisiae* transformants (see table 3). *E. coli* JM101 was used for M13 phage plaque amplification.

Growth of bacteria occurred at 37 °C whether in liquid culture or on plates. Routinely, bacteria were grown either in Luria-Bertanini (LB) medium (10 g L⁻¹ Bacto tryptone, 5 g L⁻¹ Yeast Extract, 10 g L⁻¹ NaCl) or 2 X YT medium (10 g L⁻¹ Bacto Yeast Extract, 16 g L⁻¹ Bacto Tryptone, 10 g L⁻¹ NaCl). When large harvests were required, cultures were made in Terrific broth (TB) medium (12 g Bacto Tryptone, 24 g Bacto Yeast extract, 4 mL glycerol autoclaved in 900 mL to which 100 mL 0.17 M KH₂PO₄, 0.72 M K₂HPO₄ was added) (Tartoff and Hobbs, 1987).

Bacteria were stored at -70 °C in a solution of 20 % (v/v) glycerol in growth medium. One volume of exponential culture was added to one volume of medium with 40 % (v/v) glycerol and flash frozen in a bath of methanol dry ice. Liquid cultures were inoculated from frozen stocks with scrapings carried out with the tip of a sterile toothpick. In order to maintain selection of transformants, 30-60 µg mL⁻¹ ampicillin (Ap) was included in culture media.

B. Yeast Strains: Growth and Storage conditions

Yeast strains and transformants (table 4) were grown at 30 °C either on YEA medium (0.5% (w/v) Bacto Yeast extract, 3% (w/v) glucose, and 75 mg mL⁻¹ adenine sulfate) or YNB (minimal medium) (0.67% (w/v) Bacto Yeast Nitrogen Base without amino acids (Difco), 2.0% (w/v) carbon source and supplemented with necessary amino acids). Carbon sources included either glucose, galactose or ethanol.

Yeast strains and transformants were stored at - 70 °C in solutions of 20% (w/v) glycerol in LB, YNB or YEA in a manner similar to bacterial strains and transformants (section II A).

C. Vector description

1. Bacteriophage

M13mp18 and mp19 (7.2 kb) (Viera and Messing, 1982; Messing and Viera 1982) based constructs XLH5 1,1X,2,2X (Rutledge *et. al.*, 1988)(table 4) were used to express XLH5 in *E. coli* JM103 under transcriptional control of the *E. coli lac Z* promoter for β -galactosidase. These vectors were developed from the ssDNA bacteriophage M13 and contain the N-terminal portion of the *lac Z* gene which expresses the α -peptide of β -galactosidase, capable of complementing a *lac* mutated host, to produce functional β -galactosidase.

2. Phagemid

pUC 118,119 (3.2 kb) (Viera and Messing, 1987) based constructs (table 4) were also used to express XLH5 in *E. coli* JM103 under transcriptional control of the β -galactosidase promoter. These vectors also contain the N-terminal portion of the *lac Z* gene, the β -lactamase gene (Ap^r) as well as the intergenic region of M13 which allows packaging of ssDNA plasmid DNA into a viral particle by a helper phage.

Table 3. Yeast strains and transformants used in this study

STRAIN	SOURCE	GENOTYPE
5143	N.R.C.C. Culture Collection	<i>MATa ade1, leu2-3, leu2-112 his3-15 (gal1)^a</i>
5055	N.R.C.C. Culture Collection	homothallic <i>leu2</i>
46004	A.T.C.C.	<i>MATa ade1, ade2, ura1, leu2, gal1, form II rDNA</i>
^a proposed marker (this study)		
TRANSFORMED	PLASMID STRAIN	SOURCE
5143	YEp51	This study
5143	YEpH5A-2	This study
5143	YEpH5A-i2	This study
5055	YEp51	This study
5055	YEpH5A-2	This study
5055	YEpH5A-i2	This study

Table 4 Chimeric clones containing XLH5 cDNA

CLONED DNA	SOURCE	DESCRIPTION
XLH5A-1	Rutledge <i>et. al.</i> , 1988	Contains 295 nt of XLH5 cDNA truncated by 27 nt, 3' to methionine start codon in the same orientation as <i>lacZ</i> . (M13mp9 parent)
XLH5A-1X	" "	Contains XLH5 cDNA same insert as in H5A-1 but opposite orientation to <i>lacZ</i> . (M13mp8 parent)
XLH5A-2	" "	Contains 770 nt of XLH5 cDNA truncated by 18 nt, 3' to start codon in the same orientation as <i>lacZ</i> . 3' UTR contains 200 nt (M13mp9 parent)
XLH5A-2X	" "	Contains same XLH5 cDNA insert as H5A-2 but in opposite orientation to <i>lacZ</i> . (M13mp8 parent)
p8H5A-1	This study	Contains same cDNA as XLH5A-1, but cloned in <i>HindIII-EcoRI</i> site of pUC118.
p8H5A-2	This study	Contains reconstituted coding region of XLH5 cDNA + 11 nt cloned in <i>BamHI-EcoRI</i> site of pUC118.

continued next page

CLONED DNA	SOURCE	DESCRIPTION
p9H5A-1	This study	Contains same cDNA as in XLH5A-1 but cloned in <i>HindIII-EcoRI</i> site of pUC119
p9H5A-2	This study	Contains same cDNA as in XLH5A-2 but cloned in <i>HindIII-EcoRI</i> site of pUC119
YEpH5A-1	This study	Contains 295 nt of XLH5 cDNA (<i>PvuII-BamHI</i> fragment of p9H5A-2), truncated by 28 nt 3' to start codon, inserted in YEp51 (<i>Sall</i> to <i>BamHI</i>) in the same orientation as the <i>GAL10</i> promoter
YEpH5A-1X	This study	Contains cDNA as in YEpH5A-1 in an orientation opposite to the <i>GAL10</i> promoter of YEp51.
YEpH5A-2	This study	Contains 770 nt of XLH5 cDNA from p9H5A-2 (<i>BamHI</i> fragment) in the same orientation as the <i>GAL10</i> promoter of YEp51.
YEpH5A-i2	This study	Contains same XLH5 cDNA as in YEpH5A-2 cloned into YEp51 <i>BamHI</i> .

3. Plasmid

YEp51 (7.3 kb) (kindly provided to Dr. V Seligy by Dr. J. Broach) (Broach *et al.*, 1983) based constructs (table 4) were used to express XLH5 in *S. cerevisiae* under transcriptional control of the *GAL10* (uridine diphosphoglucose 4-epimerase gene) promoter. Transformation of yeast *LEU2*- strains to *LEU*⁺ is made possible by inclusion of the *LEU2* gene which codes for β -isopropylmalate dehydrogenase. The plasmid contains the origin of replication for the 2- μ m circle, the 2- μ m circle REP3 locus, which is believed to promote stability and equipartitioning of plasmid into daughter cells (Volkert *et al.*, 1989), and a 2- μ m termination site. Since YEp51 contains the β -lactamase gene and the ColE1 origin of replication, it can be selected and propagated in *E. coli*.

D. DNA characterization

1. Plasmid DNA isolation from bacteria

Small scale bacterial plasmid preparations ("mini-preps") were carried out as in Maniatis *et al.* (1982) in order to screen transformants generated from ligation or yeast plasmid recovery and/or provide template for sequencing reactions.

Contents of an overnight 1 mL culture, that included Ap, was transferred to an eppendorf tube and pelleted in an Eppendorf Micro Centrifuge. All micro-centrifugations were done at full speed for this and all other methods. The cell pellet was lysed by suspension in 100 μ L lysis buffer (50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA and 4 mg mL⁻¹ lysozyme). After incubation for five minutes at room temperature, 200 μ L of a solution of 0.2 N NaOH, 1% SDS was added to the suspension and the tube inverted three times and incubated for five minutes at room temperature. Preparations were vortexed for 10 s after addition of 150 μ L ice cold 5M potassium acetate (pH 4.8) and stored on ice for five min before clarification by micro-centrifugation for 5 min.

Nucleic acids were deproteinized by mixing samples 50 or 60 times by

inversion, with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1). Aqueous and organic phases were separated by centrifugation for 3 min. Nucleic acids in the aqueous portion were precipitated (see section D 4 a) and collected by micro-centrifugation for five min at 23 °C. The pellet was dried in a Savant sample concentrator and resuspended in 50 μ L of TE (pH 8.0) containing RNase A (Sigma) at 20 μ g mL⁻¹. The yield of DNA from a 1 mL culture varied from 20 to 50 μ g. Restriction enzyme analysis took place as described in section D 4 b.

Cesium chloride purified plasmid preparations were carried out as described by Birnboim and Doly (1979) and Maniatis *et. al.* (1982), with modifications, in order to provide large quantities of pure plasmid, which were useful when isolating DNA fragments, restriction mapping, and carrying out yeast transformation.

A one liter culture of bacteria in TB broth with Ap was obtained by progressive "scale- up" of cultures. Initially, frozen stock was used to inoculate a 10 mL overnight culture, which was in turn used to inoculate a 250 mL culture the following day and a 1 L culture overnight. Bacterial lysis involved resuspension of cells in 20 mL lysis buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) and addition of a 10X stock of lysozyme in the same buffer for a final concentration of 5 mg mL⁻¹. Cells were incubated for 10 to 15 min at 23 °C. An equal volume of 0.2 N NaOH, 2% SDS was added , and the contents mixed by gentle inversion and incubated on ice for 10 min. After addition of 15 mL of potassium acetate pH 4.8 the solution was mixed vigorously and incubated on ice for 10 min.

Chromosomal DNA and cell debris were removed from the supernatant by centrifugation for 15 min at 8K rpm at 4 °C. Plasmid DNA and RNA were precipitated from supernatant by addition of 0.6 volumes of isopropanol and incubation for 30 min at 23 °C. Fibrous nucleic acids were recovered by a 30 min centrifugation at 12 K rpm, 15 °C. The supernatant was decanted and the pellet dried and dissolved in 6.0 mL TE.

A continuous gradient was made by dissolving 1.0 g CsCl per mL solution with 0.3 mL of a solution of ethidium bromide (10 mg mL^{-1} in water) for every 10 mL CsCl/DNA solution. The mixture was transferred to a Beckman centrifuge tube (Ultraclear 16 X 76 mm) and topped with mineral oil before centrifugation at 35K for 72 hours in a 50 Ti rotor using a Beckman L8-70 ultracentrifuge.

At the end of the centrifugation DNA bands were visualized in the tubes with long wavelength ultraviolet light. The lower of the bands which corresponded to covalently closed circular plasmid DNA was collected into a 15 mL Corex tube using a 21 gauge needle. EtBr was extracted from the aqueous plasmid DNA solution by isopropanol saturated with NaCl. DNA solution and solvent were combined in a 1:1 ratio and phases were mixed by tapping of the tube. The lower aqueous phase was transferred to another tube and the process repeated until the aqueous phase was clear. Two volumes of distilled water was added to the solution and the tube topped with isopropanol to precipitate the DNA. After overnight precipitation, plasmid was pelleted by centrifugation at 8 K rpm, 30 min. Salt was removed from the DNA pellet with a 70 % ethanol wash. The pellet was dried and resuspended in TE pH 8.0.

2. DNA isolation from yeast

a) Total DNA

Total DNA was isolated from *S. cerevisiae* using modified published methods (Hoffman and Winston, 1987). Stationary yeast cell cultures (grown in YNB medium and appropriate supplements) were collected (5K rpm, 5 min) and resuspended in 0.5 mL TE pH 8.0. Cells were transferred to a 1.5 mL microfuge tube and pelleted by a 5 sec centrifugation in a micro-centrifuge. Supernatant was removed by aspiration and the pellet of cells was resuspended in residual liquid.

Cells in the microfuge tube were lysed by vortexing with 0.3g nitric acid-washed and baked glass beads, 0.2 mL lysis buffer (2% Triton X-100, 1% SDS, 100

mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 0.2 mL phenol:chloroform:isoamylalcohol (25:24:1) for 3.5 to 4.0 minutes, and 0.2 mL TE was added. Aqueous and organic layers were separated by centrifugation for 3 min. Nucleic acid precipitation from the aqueous layer involved addition of 1.0 mL 95% ethanol, inversion of the tube and incubation on ice for at least five minutes. The tube was centrifuged for two minutes, ethanol was discarded and the pellet was dried *in vacuo*.

The pellet was resuspended in 0.4 mL TE with 30 μ g RNase A (Sigma) and incubated at 37 °C for at least 15 min. DNA was precipitated by addition of 1.0 mL ethanol. Micro-centrifugation (5 min, 4 °C) followed and the pellet was washed in 0.2 mL ice cold 70% ethanol. The pellet was dried in a Speedvac lyophilizer and resuspended in 50 μ L TE and incubated for 5 min at 37 °C. Sample concentration and integrity were inspected by agarose gel electrophoresis.

b) Release of plasmid DNA

Plasmid was released from lysates of *S. cerevisiae* transformants, grown under selective conditions (Hoffmann and Winston, 1987). Cells were lysed by vortexing with glass beads in the same manner as for genomic DNA, but for 2 min without TE. Competent JM103 cells (0.1-0.2 mL) were transformed to Ap resistance with plasmid present in 5-10 μ L of the aqueous layer (section II E 1).

3. DNA Quantitation

DNA concentration was determined by reading absorbance at 260 nm on a Gilford 2600 spectrophotometer. Concentration was assessed according to the relationship, $A_{1\text{cm } 260\text{nm}} = 1$ for 50 μ g mL⁻¹ dsDNA.

4. DNA Manipulation

a) Precipitation

DNA solutions were concentrated by using ammonium acetate as a salt to improve the efficiency of precipitation. Ammonium acetate was added to 2.5 M for

solutions to be precipitated (0.5 volumes of the solution from a 7.5 M stock). Two volumes of ethanol were added to the solution and the sample incubated at -20°C . The salt of the DNA was recovered by micro-centrifugation for at least 15 min. The pellet was washed with 0.2 mL ice-cold 70% ethanol. It was dried in a Speedvac lyophilizer and resuspended in TE pH 8.0 to at least $50\text{ ng }\mu\text{L}^{-1}$.

b) Restriction endonuclease treatment

Restriction enzymes from various commercial sources (eg. Pharmacia, BRL, IBI etc.) were used to digest DNA (plasmid, genomic). Digests were carried out under conditions of ionic strength and temperature defined by Pharmacia using at least 4 units restriction endonuclease per μg DNA.

When resultant fragments were to be subsequently used with different enzymes, digestion was terminated by heat inactivation (65°C , 10 min). When reaction products were to be analyzed by agarose gel electrophoresis, 1/10 reaction volume stop solution (50% (v/v) glycerol, 0.1 M EDTA (pH 8.0), 1% (w/v) SDS and 0.1% (w/v) bromophenol blue) was added.

c) Recovery of fragments from plasmid DNA

DNA of desired size was recovered from a restriction digest after electrophoretic separation of reaction products in an agarose gel containing ethidium bromide. DNA fragments were visualized using a hand-held, long wavelength UV illuminator. Agarose gel slices containing desired DNA were excised and placed in an electroelution apparatus (IBI). Fragments were recovered by electrophoresis of DNA into a dye-salt cushion (7.5 M $\text{NH}_4\text{Acetate}$, 0.01% bromophenol blue) at 80 V. Electrophoresis was monitored by fluorescence of gel slices illuminated with UV light. When DNA was no longer present in the gel slices, the salt cushion was collected and the DNA therein was ethanol precipitated.

d) Ligation

Cloning experiments involved the joining of DNA (insert) fragments with

either protruding blunt or cohesive ends with various DNA vectors. Ligations involving cohesive ends required the use of a vector to insert ratio = 0.5 and overnight incubation at 8 °C in a reaction volume of 20 μ L containing 0.5 units T4 DNA ligase (Pharmacia) in 1 X ligase buffer (25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP). Blunt end ligations involved the use of a vector to insert ratio = 3.0 in a 20 μ L volume containing 2 units T4 DNA ligase in 1 X ligase buffer with a 2 to 4 hr incubation at 23 °C. Both types of ligation reactions were terminated by the addition of 1 μ L of 0.5 M EDTA.

Synthetic oligonucleotides (35-39 bases in length) were designed to link the 5' end of partial XLH5 cDNA and the vector. These linker sequences were heat-denatured at 70 °C for five min and reannealed to form duplexes by transfer to a beaker containing water at 65 °C. The bath was allowed to cool to room temperature to promote annealing of oligomers to give duplex linkers. Ligation was carried out with a vector to insert to linker ratio of 1:2:12.

Chimeric vectors were recovered by transformation of *E. coli* JM103 to Ap resistance with plasmid in 5-10 μ L of the ligation reaction volume (section II E 1).

e) Creation of blunt ended DNA

Blunt ended DNA molecules were created by filling in the sequence of 3' recessed ends with T4 DNA polymerase. DNA (approx 22 ng) was mixed with 33.3 μ M each of dGTP, dCTP, dATP and 16.6 μ M dTTP in 60 μ L containing 33 mM Tris-acetate pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 5 μ M DTT, 6 μ g bovine serum albumin and T4 DNA polymerase (1 unit). The reaction occurred at 37 °C, 30 min. Unincorporated nucleotides were removed by spun column chromatography (section D 8 f).

f) Radiolabelling of DNA probes

dsDNA was radiolabelled by nick translation (Rigby *et. al.*, 1977; Maniatis *et. al.*, 1982). In a final volume of 48 μ L, DNA (0.5 μ g) was combined with 1.5 μ M each

of dGTP, dCTP, dTTP, 75 μCi [α ^{32}P] dATP in 50 mM Tris-HCl, 10 mM MgSO_4 , 100 μM DTT, 50 $\mu\text{g mL}^{-1}$ BSA in 48 μL . Nicking of dsDNA was initiated by addition of 1 μL of a 10^{-4} dilution of DNase I and incubated at 23 $^\circ\text{C}$ for 3 min. Repair was initiated by addition of DNA pol I (10 units) and the reaction was incubated at 16 $^\circ\text{C}$ for 1 h. The reaction was terminated by addition of 5 μL 20% SDS and 45 μL TE (pH 8.0).

dsDNA was also radiolabelled by random-primed (Feinberg and Vogelstein, 1983) incorporation of [α ^{32}P] dATP using commercially prepared ssDNA hexanucleotide primers (Boehringer Mannheim). DNA (0.1 -0.5 μg in 1 μL) and primers (2 μL) were denatured (10 min at 100 $^\circ\text{C}$) and cooled on ice. Reaction involved addition of 25 nM dCTP, dGTP, dTTP, 50 μCi [α ^{32}P] dATP (800 Ci/mmol or 3000 Ci/mmol) and 2 units of labeling grade Klenow enzyme in a volume of 20 μL . Reaction was carried out for 30 min at 37 $^\circ\text{C}$ and stopped by heating at 65 $^\circ\text{C}$ for 10 min.

Oligonucleotides were phosphorylated at the 5' end as described by Maniatis *et. al.* (1982). Oligonucleotide (60 ng) was combined with 50 μCi [γ ^{32}P]-ATP (3000Ci/mmol, 10 $\mu\text{Ci}/\mu\text{L}$) in 50 μL containing 50 mM Tris-HCl pH 7.6, 10 mM MgCl_2 , 5 mM DTT, 100 μM spermidine hydrochloride, 100 μM EDTA and 20 units of T4 polynucleotide kinase. The reaction was incubated 37 $^\circ\text{C}$, 30 min. Termination of the reaction (65 $^\circ\text{C}$, 10 min) was followed by spun column chromatography through Biogel P6 (Biorad).

Unincorporated [^{32}P] dNTPs from labelling reactions were removed from labelling reactions by centrifugation through Sephadex G-50 (Pharmacia) or Biogel P6 (Biorad) packed in disposable 1 mL syringes (Maniatis *et. al.*, 1982). Stocks of both Sephadex G-50 and P6 were equilibrated in TE (pH 8.0), autoclaved, cooled to room temperature and stored at 4 $^\circ\text{C}$.

Columns were made using 1 mL syringes plugged with a small amount of

siliconized glass fibre paper. Columns were held in place using a glass centrifuge tube. Chromatography beads were added to the column with a sterile pasteur pipette and packed by centrifugation at 2.5 K rpm for 4 min. This step was repeated until the packed bed volume was 0.9 mL.

Radiolabelled DNA was applied to the column in a volume of 100 μ L and centrifuged as above. The effluent, containing the probe was collected in a decapped microfuge tube placed at the bottom of the tube. The probe was stored at -20 °C until further use.

Percentage incorporation of radioisotope into DNA was determined by spotting 1 μ L of reaction solutions (before and after spun column chromatography) onto Whatman glass microfibre filters and calculation of total cpm, when Cerenkoff radiation was detected in a Beckman LS 7000 liquid scintillation counter.

5. DNA Analysis

a) Nucleotide Sequencing

The following is an outline of how direct sequencing of ds plasmid DNA was carried out by the chain termination method (Sanger, Nicklen and Carlson, 1977) using genetically modified variants of bacteriophage T7 DNA polymerase in the sequencing reactions (Sequenase (Tabor and Richardson, 1987) or Sequenase version 2.0).

(1) Template preparation

Plasmid (1-2 μ g) was denatured with 1 μ L 10 mM EDTA and 4 μ L 1N NaOH and water in a volume of 20 μ L for five minutes at 23 °C and precipitated with 2.5 volumes of EtOH. After washes with chilled 70% EtOH, the pellet was dried under vacuum and dissolved in 2.0 μ L containing the appropriate primer, 2.0 μ L of 5 X sequencing buffer (200 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 250 mM NaCl), 6.0 μ L deionized H₂O. Annealing took place at 37 °C for 15-20 min.

Since typical reactions involved reading sequences within 400 nucleotides of

the primer, the dGTP labelling mix (7.5 μ M dGTP, 7.5 μ M dCTP, 7.5 μ M dTTP) was diluted five fold with distilled water. In order to deduce sequences where compressions were a problem, dITP labelling mix (15 μ M dITP, 7.5 μ M dCTP, 7.5 μ M dTTP) was used instead. Since dITP potentiates pausing artifacts, dITP reactions were run alongside dGTP reactions.

(2) Labelling and termination reaction

Labelling reactions were executed by adding to the template-primer mixture: 1.0 μ L of 0.1 M DTT, 2.0 μ L of diluted labelling mix (above), 1.0 μ L of [α - 35 S]-dATP and 2.0 μ L diluted sequenase (1:8 in ice cold distilled water). The reaction occurred at room temperature for 5 min.

Four separate termination mixes, each with a ddNTP where N=A,C,G or T, matched with the labelling mix used in the reaction (dGTP or dITP), were prewarmed to 37 °C in separate tubes. Each mix contained 80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 50 mM NaCl and 8 μ M ddNTP.

Each termination tube received 3.5 μ L of the labelling reaction. Termination reactions occurred at 37 °C for 3 min and were stopped at 65 °C for 10 min. Samples were dried under vacuum and dissolved in 4 μ L of sequencing "stop mix" (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF).

(3) Gel electrophoresis

Labelled fragments from sequencing reactions were electrophoretically separated in denaturing polyacrylamide gels of uniform or non-uniform thickness. Electrophoresis was carried out using a Bethesda Research Laboratories Model S2 sequenator containing 1 X TBE buffer using the following settings: 60 W, 60 mA and 1800 V.

Uniform thickness gels (Carlson and Messing, 1984) of 6% acrylamide were prepared by heating a mixture of 15 mL 40% acrylamide (a filtered solution of 38 g

acrylamide, 2 g bisacrylamide in 100 mL), 10 mL TBE buffer and 48.1 g of electrophoresis grade urea in a total volume of 100 mL with water. After filtration through Whatman filter paper, polymerization was initiated by addition of 40 μ L TEMED and 400 μ L APS and completed by one hr. After 30 min of pre-electrophoresis, 2 μ L of sequencing mix, which had been boiled for 2 min, was loaded onto the gel and electrophoresed until the bromophenol blue dye had reached the gel bottom, which enables one to read a sequence of approximately 135 nt. The gel was then removed from the apparatus and fixed in 5% methanol, 5% acetic acid for 10 min before mounting on Whatman 3MM paper and drying.

Wedge gels (Carlson and Messing, 1984) were cast with the aid of spacers 0.4 mm thick at top and 1.2 mm thick at bottom. The gels, composed of 5% acrylamide, were made by mixing and heating of a 150 mL solution containing 18.75 mL 40% acrylamide, 72 g urea and 7.5 mL of 10 X TBE. The solution was filtered and polymerized for one hr, following addition of 55 μ L TEMED and 550 μ L APS. After 30 min of pre-electrophoresis, the gel was loaded and electrophoresed until the bromophenol blue dye had reached the gel bottom. The gel was fixed in 5% methanol, 5% acetic acid for 30 min before mounting and drying.

(4) Autoradiography

The dried gel was covered with Saran wrap and placed inside a cardboard Kodak cassette. Kodak XRP film was placed against the film inside the cassette, and the combination was placed between two pieces of ply-board. Lead weights were placed on top of the boards to maintain contact. Film development occurred after 3-4 days.

b) DNA electrophoresis in agarose gels

dsDNA was electrophoresed in agarose (Seakem) gels of 0.7-1.5% (w/v) containing 1 X TAE buffer (25 mM Tris, 25 mM acetic acid, 10 mM EDTA pH 7.8) and 0.5 μ g mL⁻¹ ethidium bromide. Samples were electrophoresed at approximately

33 V cm⁻¹.

ssDNA was electrophoresed in the absence of ethidium bromide in agarose (Seakem) gels of 0.6% (w/v) containing 1 X TBE buffer (89 mM Tris-borate, 89 mM Boric acid, 2 mM Na₂EDTA pH 8.0). Gels were stained after electrophoresis by soaking in 0.5 µg mL⁻¹ ethidium bromide for 5 min and destained for 15 min in 1 X TBE.

Photographs of gels were made in the presence of short wave UV light (310 nm) from a transilluminator with a Polaroid camera fitted with a red filter and type 52 film (Polaroid corp.).

c) Southern hybridization

(1) Transfer of DNA in agarose gels onto nylon membrane

dsDNA was transferred from agarose gels onto nylon membranes by the alkaline transfer method of Reed and Mann (1985). DNA was acid-nicked by soaking the gel in 0.25 N HCl for ten min before neutralization in 0.3 M NaOH, 1.5 M NaCl followed. DNA was blotted onto nylon by passive diffusion for 4 to 6 h in the presence of 0.3 M NaOH, 1.5 M NaCl on a blotting apparatus. Both the blot and gel were neutralized by a 10 min soaking in 2 X SSC. DNA was immobilized by baking the blot at 80 °C for 1 h. Efficiency of transfer was determined by staining of the gel in 5 µg mL⁻¹ EtBr and visualization of DNA with UV light.

(2) DNA/DNA hybridization

Southern blots and colony hybridizations were prehybridized and hybridized at 65 °C in solution consisting of 1 M NaCl, 1.0 % SDS and 100 µg mL⁻¹ heat-denatured salmon sperm DNA in heat sealable 2 mil thickness polyester bags (Dazey corp.). After prehybridization for at least 1 hr, the radiolabelled probe was heat-denatured at 100 °C for 10 min and added to the hybridization solution for a probe concentration of greater than 10⁶ cpm mL⁻¹. Hybridization occurred for 16-24 hours.

Filters were washed under various conditions. Medium stringency washing was done in 2 X SSC, 0.1% SDS for fifteen minutes with constant agitation. Three washes, each of 20 min, took place in 2X SSC, 0.1 % SDS at 65 °C. Filters were mounted on Whatman 3MM paper and wrapped in plastic. They were exposed to Kodak XRP or XAR film with intensifying screens for durations of up to 1 week at -70 °C.

In order to remove probes and to reuse southern blots for hybridization with other probes, blots were boiled for 20 min in 0.1% SDS solution. Absence of radioactivity on blots was confirmed by autoradiography.

d) Sequence alignments

Nucleotide sequence alignments were carried out using the FASTA software package of NRCC.

E. Transformation

1. Bacterial

Competent *E. coli* JM103 cells were prepared as outlined in Chung and Miller (1988) and Chung *et. al.* (1989). Cells were grown in LB broth to early exponential phase (OD 600 nm 0.3-0.4). Cells were pelleted gently at 3K rpm for 5 min in a clinical centrifuge and made competent by resuspending and incubating them in 1/10 the culture volume of ice-cold TSS broth ((10% (w/v) PEG 3350, 5% (w/v) DMSO, 30 mM Mg²⁺ in 1 X LB broth) and cells were incubated on ice for approximately 10 minutes. Transformation of competent cells to Ap resistance was achieved by the addition and gentle mixture of a 100 µL aliquot of cells in TSS broth to at least 20 pg plasmid DNA from either a mini-prep (section D1), a yeast plasmid isolation (section D2(b)), a ligation product (section D4(d)) or a large-scale preparation. Inter-trial transformation efficiencies were compared by using 29 pg of covalently closed CsCl purified pUC119 DNA as a control. After incubation of the

cell/DNA mixture on ice for 20 minutes, 0.9 mL of TSS containing 20 mM glucose was added to the mixture and the tube incubated with agitation for one hour. Transformed bacteria were selected by plating 100 μ L aliquots on fresh plates containing 30-60 μ g mL⁻¹ Ap.

2. Yeast

a) Spheroplasting

Yeast transformants were obtained by a spheroplasting method described by Beggs (1978). The yeast host strain was grown on YPD medium (100 mL) to an approximate density of 2×10^7 cells mL⁻¹ and harvested (5K, 5 min, Beckman AccuSpin FR). Cells were washed once with sterile distilled water and resuspended and incubated (30 °C, 10 min) with gentle shaking in 10 mL SED (1M Sorbitol, 25 mM EDTA pH 8.0, 50 mM DTT). After collection, as above, cells were washed once in 10 mL SORB (1M Sorbitol) and resuspended in 10 mL SCE (1M Sorbitol, 0.1 M sodium citrate pH 5.8, 0.01 M EDTA).

Spheroplasts were generated by addition of 0.1 mL Glusulase (Endo Laboratories, Inc.) to the slurry and incubation at 30 °C for 30 min with periodic swirling. Spheroplasting was monitored microscopically by the observation of "ghosts" at 400 X phase contrast following the addition of 10 μ L cells into a drop of 5% SDS.

Spheroplasts were collected by gentle centrifugation at 2.5K rpm for 3 min and washed twice with 10 mL SORB. A wash in STC followed (1M Sorbitol, 10 mM CaCl₂, 10 mM Tris-HCl (pH 7.5)). The pellet of cells was suspended in 0.5 mL STC and divided in 0.1-0.2 mL aliquots in 15 mL disposable tubes containing 5-40 μ g DNA in 1-10 μ L. After incubation of the mixture at room temperature for ten minutes, 1.0 mL PEG (20% PEG 4000, 10 mM CaCl₂, 10 mM Tris-HCl (pH 7.5)) was added to each tube. Tubes were left at room temperature for ten minutes and centrifuged (2.5 K rpm, 5 min) and resuspended in 150 μ L SOS (10 mL 2M

Sorbitol, 6.7 mL YPD, 0.13 mL CaCl_2 , 0.5 mL leucine and 3.17 mL water). Cells were incubated at 30 °C for 20 min, suspended in warm top agar (per 100 mL: 18.2 g sorbitol, 2 g agar, 0.67 g Difco yeast nitrogen base (without amino acids), 2 g glucose, 75 mg mL^{-1} amino acids as required) and plated on selective media (per liter: 182 g sorbitol, 20 g agar, 6.7 g Difco yeast nitrogen base (without amino acids), 20 g glucose, 75 mg mL^{-1} adenine).

b) Alkaline cation method

Yeast transformants were also generated by the alkaline cation method (Ito *et. al.*, 1983; Rose, 1987). Cells from an overnight starter culture in 10 mL YEPD were diluted 1:50 in 100 mL fresh YEPD in a 250 mL erlenmeyer flask. Cells were harvested at mid-logarithmic phase ($1-2 \times 10^7$ cells mL^{-1}) by centrifugation at 5K rpm for 5 min. Cells were resuspended in one-half culture volume of 0.1 M LiCl in TE (pH 7.5) and incubated at 30 °C for 1 hour. After harvest, cells were resuspended in 1 mL of 0.1 M LiCl in TE (pH 7.5) and 100 μL cells were aliquoted into eppendorf tubes. After incubation (30 °C, 30 min), 0.7 mL 40% (w/v) PEG 3350, 0.1 M LiCl in TE (pH 7.5) was added to cells, which required thorough mixing. Cells were incubated at 30 °C for 1 hour and then heat shocked (42 °C, 5 min). Cells were pelleted for 5 s in a micro-centrifuge and washed twice in 0.5 mL TE. Transformed cells were resuspended in 0.4 mL H_2O and plated on selective plates.

c) Screening by Colony hybridization

Putative yeast transformants were analyzed by a modification of published methods (Carter *et. al.*, 1987). Colonies were replica plated onto a master plate and on sterile nylon filters placed on the surface of selective plates. After 48 h incubation, filters were removed and allowed to dry. Filters were placed colony side up on a pool of SED on saran wrap and incubated for 15 min. Filters were transferred to Whatman 3MM paper saturated with SCE plus 1% (w/v) Glusulase.

Spheroplasting was carried out in a petri plate at 37 °C for 1 hr. Spheroplasts were transferred to a pool of 0.5 M NaOH at 23 °C for 7 min. Neutralization of filters involved soaking in each of 0.5 M Tris-HCl pH 6.9, 10 X SSC, and 0.5 M Tris-HCl pH 7.5, 10 X SSC each for 4 min and in 5 X SSC twice for 2 min. Filters were dried on 3MM paper (30 min) and baked at 80 °C for 1 hr.

F. Yeast RNA isolation and analysis

1. Isolation

Yeast RNA was isolated using modifications of established techniques (Glisin *et. al.*, 1984; Chirgwin *et. al.*, 1979). To 0.3-1.0 gram pellet (wet weight) of frozen cells the following were added: an approximately equal volume of acid washed and baked glass beads (0.5 mm diam), 2.5 mL guanidinium isothiocyanate buffer (4M guanidinium isothiocyanate, 25 mM sodium citrate pH 7.0, 1mM EDTA pH 7.0), 25 μ L β -mercaptoethanol, 40 μ L antifoam A, 12.5 μ L sarkosyl. The mixture was vortexed at high speed (2 min).

Cell debris was removed by centrifugation (10 K rpm, 5 min). The supernatant was layered on a 2.0 mL cushion of 5.7 M CsCl, 0.01 M EDTA (pH 7.5) in a clear SW50.1 ultracentrifuge tube. Ultracentrifugation followed in a Beckman L8-70 Ultracentrifuge (18 °C, 30 K rpm, 16 hr). Supernatant and debris were removed from tubes, by aspiration, after centrifugation and the tubes rapidly inverted to allow supernatant to drain away from the RNA pellet at the bottom. Tubes were retained in an inverted position for 0.5 h.

The RNA pellet was transferred to an eppendorf tube after ethanol was added dropwise to the pellet. Ethanol was evaporated from the pellet by drying the sample *in vacuo*. RNA was dissolved in diethyl pyrocarbonate treated water by subjecting the aliquot to several cycles of -70 °C to +65 °C. Any residual protein remaining was removed from samples through the use of phenol:chloroform:isoamylalcohol (25:24:1). Samples were concentrated by ethanol precipitation.

Size and quality of RNA was confirmed by horizontal mini-gel electrophoresis. A small aliquot of each sample (1-2 μL) was combined with 2 μL tracking dye (400 μL DMSO which contained 40 μL of a solution of 1% xylene, 1% bromophenol blue, 1% orange G). Samples were electrophoresed in a 2.0% agarose, 0.025 M citrate gel (pH 6.5) at 60-65 V for at least one hour. In order to visualize RNA, the gel was immersed in a solution of 5 $\mu\text{g mL}^{-1}$ EtBr for at least 15 min. The gel was destained for at least fifteen min in ddH₂O and photographed under UV light.

Absorbance readings at 260 nm were obtained on a Gilford 2600 Spectrophotometer. Sample concentration was assessed according to the relationship of $A_{1\text{cm } 260\text{ nm}} = 1$ for 40 μg RNA (Maniatis *et. al.*, 1982).

Yeast poly(A⁺) mRNA was selected through the use of oligo(dT) cellulose chromatography (Aviv and Leder, 1972), as described in Sombrook *et. al.* (1989). A slurry of oligo(dT) cellulose (Boehringer Mannheim) was equilibrated in 0.1 N NaOH and poured into a sterile column (Biorad). The matrix was washed with column loading buffer (20 mM Tris-HCl (pH 7.6), 0.5 M NaCl, 1 mM EDTA (pH 8.0, 0.1% SDS) until the pH of the effluent was less than 8.0.

Total mRNA, dissolved in sterile water, was heated to 65 °C for 10 min and then combined with an equal volume of 2 X column loading buffer, was applied to the column and the eluate collected. One volume of 1 X column loading buffer was applied to the column, after all the RNA solution had entered, and the eluate was collected. All eluted solution was pooled, heated and reapplied to the column. New eluate, which contained nonpolyadenylated RNA, was collected. Five column volumes of 1 X column loading buffer was applied to the column and OD₂₆₀ readings were measured for each 1 mL fraction collected.

Poly(A⁺) mRNA was collected by elution with 10 column volumes of elution buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), 0.05% SDS). Fractions, collected in 0.1 mL volumes, were precipitated with ethanol (as above) and

dissolved in 5 μ L DEPC treated H₂O. Yield of poly(A⁺) mRNA was approximately 28 μ g per mg total RNA.

2. Analysis

a) Formaldehyde gel electrophoresis

Electrophoresis of RNA was carried out in a 1% agarose, 0.66 M formaldehyde gel in running buffer consisting of 0.2 M morpholinepropanesulfonic acid pH 7.0, 50 mM sodium acetate, 10 mM EDTA (pH 8.0) (Maniatis *et. al.*, 1982; Fournery *et. al.*, 1988). Each 10 μ g sample of RNA was dissolved in 4.5 μ L DEPC treated water, 2.0 μ L 10 X running buffer, 3.5 μ L formaldehyde, 10.0 μ L formamide. After heat denaturation (65 °C, 10 min), 2.0 μ L sterile loading buffer (50% glycerol, 1mM EDTA, 0.4% BPB, 0.4% XC) was added and samples were loaded on the gel. Typically, electrophoresis was for 18 hr at 30V. At end of the run, control lanes were excised and stained in EtBr.

b) Northern hybridization

(1) Transfer of denatured RNA onto nylon membrane

Electrophoresed RNA was electroblotted onto a nylon membrane (either Nytran, Schleicher and Schuell or Hybond, Amersham), as described in the manufacturers protocols for Nytran membrane. Transfer was done in a Hoefer model TE50 apparatus (transfer buffer consisting of 12 mM Tris, 6 mM sodium acetate, 0.3 mM EDTA at pH 7.0) maintained at 10 V for 1 hr, followed by 20 V for 2 hr. The northern blot was air dried and baked at 80 °C for 1 hr.

Total RNA samples were also immobilized onto nylon membrane with a Schleicher and Schuell Minifold II apparatus. Serial dilutions of RNA from different time points were established in 100 μ L RNase free water. Samples were denatured by addition of 300 μ L of 6.15 M formaldehyde and 10 X SSC and incubation at 65 °C for fifteen minutes. Samples were pipetted through sample wells and the wells rinsed with 400 μ L of 10 X SSC. The filter was dried and baked in a vacuum oven

for one hour in order to immobilize the nucleic acid.

(2) RNA/DNA hybridization and quantitation of autoradiograms

Northern blots were prehybridized and hybridized at 42 °C according to conventional methods. Prehybridization occurred for 1 hr in a 2 mil thick heat sealable polyester bag in 50% (v/v) deionized formamide, 5 X SSC, 5 X Denhardt's solution, 50 mM sodium phosphate (pH 6.5), 0.1 % SDS, 100-250 $\mu\text{g mL}^{-1}$ heat denatured salmon sperm DNA, 50 $\mu\text{g mL}^{-1}$ bacterial or yeast tRNA. Prehybridization for at least one hr (up to 6 hr) was followed by addition of heat denatured probe (100 °C, 10 min) to the bag. Probe concentration was $\geq 10^6$ cpm mL^{-1} . Hybridization occurred for 16-24 hr.

Moderate stringency washes involved circulation of 2 X SSC, 0.1 % SDS over filters at 23 °C for 15 min, followed by agitation of filters in 2 X SSC, 0.1% SDS at 65 °C.

Quantitation of autoradiograms took place on a Molecular Dynamics computing densitometer model 300A according to manufacturer's instructions.

c) Determination of the 5' end of transcripts by primer extension

Primer extension (Calzone *et. al.*, 1987) was used to determine the 5' termini of XLH5 transcripts produced in *S. cerevisiae*. Total mRNA (40 μg) samples, dried and resuspended in 10 μL DEPC treated H_2O , were made to 10 mM methylmercury and incubated (37 °C, 10 min). End-labelled primer (8 ng oligo 5D24R) and 2 μL β -mercaptoethanol (350 mM) were added, and the mixture was heated at 65 °C for 5 min. Primer and RNA were annealed by cooling of the mixture to 37 °C in a water bath over at least 20 min.

Annealed RNA and primer were combined with 1 mM each of dATP, dCTP, dGTP, dTTP, 25 units human placental RNase inhibitor (Amersham), 42 units of reverse transcriptase (Molecular Genetic Resources Super RT) in 50 μL containing 50 mM Tris-HCl (pH 7.6), 60 mM KCl, 10 mM MgCl_2 . Samples were incubated at

37 °C for 1h.

RNA was removed by treatment with 2.0 μ L 1.0 M NaOH and incubation at 65 °C for 1hr. The sample was then neutralized (20 μ L 1.0 M Tris-HCl (pH 7.5) and applied to a P6 spun column (section II D 8 d). Eluate was dried and dissolved in 4 μ L of sequencing "stop mix". Primer extended products were analyzed either on 8% polyacrylamide sequencing gels alongside ladder consisting of sequenced YEp51 derived constructs using oligo 5D24R or on 5% acrylamide denaturing gels alongside marker consisting of end-labelled denatured *Hinf*I digested pBR322.

G. Protein isolation and analysis

1. Isolation

a) Lysine rich histone extraction from X. laevis

African clawed toads were terminally anesthetized in 0.1% (w/v) MS-222 solution. The pericardial cavity was opened, the heart exposed and bathed in saline with heparin (10 mM Tris-HCl pH 7.8, 0.1 mg mL⁻¹ heparin). Toads were bled slowly over a half hour by progressively severing blood vessels connected to the heart and dilution of the blood, which pooled in the cavity, with heparinized saline. Collected blood/saline was centrifuged to recover the erythrocytes and washed with saline at 3 K rpm for 1 min in a clinical centrifuge.

Histones were purified from erythrocytes by acid extraction. Perchloric acid was added to make 5% final (w/v) and the sample was homogenized in a Dounce homogenizer for fifteen minutes. Perchloric acid soluble proteins were removed from insoluble debris by centrifugation. This and all subsequent centrifugation was done at 10 K rpm, 10 min. Protein was precipitated by addition of 1/4 volume of 100% trichloroacetic acid and incubating at 23 °C for 30 min.

The pellet was washed in acid acetone (0.2% (v/v) 12 N HCl in acetone) to remove TCA followed by a wash in acetone. The pellet was dried under a stream of nitrogen and then suspended in 5 mL distilled H₂O. Samples of the solution, frozen

at -70 °C in eppendorf tubes, were lyophilized and suspended in Laemmli buffer.

b) Intracellular protein extraction from *E. coli* and *S. cerevisiae*

Pelleted snap frozen *E. coli* cells were suspended in 100-200 μ L Laemmli buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 5% β -mercaptoethanol, 10% glycerol, and 0.005% bromophenol blue) and lysed by three successive 5 min cycles of heating (100 °C) and 'snap' freezing by immersion of sample into a methanol dry ice bath.

Yeast intracellular proteins were extracted from pelleted snap frozen cells by combining cells with an equal volume of glass beads and two volumes of cracking buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1 mM PMSF, 1 mM DTT) and vortexing at high speed for 2 min. Yeast cell contents were released by alternate 2 min cycles of vortexing cells in buffer at high speed and cooling of lysate on ice. Cell debris was removed by centrifugation (5K, 5 min). An equal volume of 2 X Laemmli buffer was added to the supernatant and the mixture was boiled (100 °C, 5 min). After remaining cell debris was pelleted by centrifugation, the supernatant was stored at -20 °C.

2. Analysis

a) SDS-PAGE

Assessment of the nature of heterologous XLH5 expression involved electrophoretic separation of intracellular protein samples by SDS polyacrylamide gel electrophoresis (Laemmli, 1970) using a mini-PROTEAN II dual slab cell (Bio-rad).

Gels were cast with a 13% separating and a 7% stacking gel. Acrylamide stock solutions of 29.2% w/v acrylamide / 0.8% N,N'-methylenebisacrylamide or 0.8% piperazine diacrylamide were used for gel preparation. The composition of the separating gel was 375 mM Tris-HCl (pH 8.3), 0.1% SDS and 0.04% w/v APS. The stacking gel contained 120 mM Tris-HCl (pH 6.8), 0.1% SDS and 0.06% APS.

TEMED at a concentration of 0.15% v/v was used to initiate gel polymerization.

Samples were electrophoresed alongside low range protein molecular weight standards (BRL): insulin (M_r 3 kDa), bovine trypsin inhibitor (M_r 6.2 kDa), lysozyme (M_r 14.3 kDa), β -lactoglobulin (M_r 18.4 kDa), bovine carbonic anhydrase (M_r 29 kDa), ovalbumin (M_r 43 kDa).

Electrophoresis was done at 200V, 45 min in electrode baths containing 25 mM Tris-HCl pH 8.3, 0.1% SDS, 192 mM glycine. Proteins in gel were stained with Coomassie Blue R (0.1 % (w/v) in 40% methanol, 10% acetic acid) and destained with 40% methanol, 10% acetic acid. Gels were dried and stored using BioGelWrap (BioDesign, Inc.)

b) Immunodetection

Electrophoresed proteins in SDS PAGE gels were transferred to nitrocellulose (0.45 μ m, Schleicher and Schuell) in the manner outlined by Shay *et al.* (1988) using a mini-trans-blot electrophoretic transfer cell (Bio-rad). The gel was incubated in half-strength Laemmli reservoir buffer (0.01% (w/v), 12.5 mM Tris, 96 mM glycine). Transfer was conducted in half-strength Laemmli reservoir buffer in an electroblot apparatus at 20 V for 1.5-2 hr. Degree of transfer of proteins was assessed by staining of gel with Coomassie Blue R.

Anti-XLH5 polyclonal antibody (supplied by Dr. J. Neelin and described in appendix 4) was cross reacted either to intracellular proteins extracted from *E. coli* or *S. cerevisiae* as required. Bacterial lysate was prepared by growth of an overnight culture of JM103 in 100 mL 2YT medium. Cells were harvested (4K rpm, 5 min in a bench top centrifuge). Cells were suspended in 10 mL TBS (50 mM Tris HCl (pH 7.5), 200 mM NaCl) and the slurry aliquoted into microfuge tubes. Bacterial cells were lysed in TBS by three successive cycles of boiling and 'snap' freezing. Cell debris was removed by high speed centrifugation. Yeast lysate was prepared by growth of a culture to mid-log in 100 mL YEPD medium. Cell harvest and lysis was

similar to above.

An optimized concentration of anti-XLH5 antibody, for visualization of a *X. laevis* LHE (5% perchloric acid extract of erythrocytes, supplied by Dr. Neelin), was determined by carrying out the immunodetection regime, outlined below, on various dilutions of LHE (0-50 ng) either spotted or electroblotted onto nitrocellulose. An appropriate concentration of primary antibody was determined by diluting anti-XLH5 antibody over several orders of magnitude (1:100 to 1:500 000), carrying out the reaction with each concentration and observing reaction appearance. An appropriate concentration of secondary antibody, to use with the concentration of primary antibody, was determined by carrying out reactions with several dilutions of secondary antibody ($0.1 - 2 \mu\text{g mL}^{-1}$ goat anti-guinea pig IgG).

Lysate preparations (1-2 mL) were cross-reacted with diluted XLH5 antiserum (1:50 000 in TBS) for 1h at 23 °C. Non-specific binding sites on the immunoblot were blocked in TBST (TBS with 0.05% Tween-20) and 1% (w/v) BSA for at least 60 min. All of the following steps were carried out at 23 °C using a rocking platform. The electrophoretic blot was bathed in diluted anti-XLH5 anti-serum (refer to above). Two 15 min washes in TBST followed. Membranes were bathed in $1 \mu\text{g mL}^{-1}$ Biotin-SP-Affinipure goat anti-guinea pig IgG (H+L) (Jackson ImmunoResearch Laboratories Inc.) in TBST (30 min). Following removal of excess biotinylated secondary antibody, by two 15 min washes in TBST, incubation in $0.08 \mu\text{g mL}^{-1}$ streptavidin-alkaline phosphatase conjugate (BRL) in TBST occurred for 30 min. Excess conjugate was removed with two 15 min washes in TBS buffer. Prior to colorimetric reaction, membranes were bathed in AP buffer (0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM MgCl_2) for 15 min. Detection of secondary antibody took place by bathing the filter in 0.16 mg mL^{-1} 5-bromo-4-chloro-3-indoyl phosphate (Bio/Can) and 0.33 mg mL^{-1} nitroblue tetrazolium (Bio/Can) for up to 4 min in AP buffer. The reaction was stopped by immersion of the membrane in distilled

deionized water.

c) M_r determination for XLH5 and expression products

Due to high charge density, lysine-rich histones, such as XLH5, do not exhibit electrophoretic mobilities in SDS-PAGE that reflect their molecular weights. In this thesis, for comparative purposes, M_r values were determined for the XLH5 expression products and authentic XLH5 by interpolation from a plot of $\log M_r$ of protein standards (above, section G2a) versus electrophoretic mobility. These interpolated M_r values appear in quotations in order to emphasize that they reflect mobilities compared to protein standards with 'uniform' charge densities.

d) Sequence alignment

Amino acid sequence alignments were carried out using FASTA software.

III. RESULTS

A. Characterization of cloned XLH5 and expression in *E. coli*

1. M13mp8,9 vector

The first approach taken towards the analysis of XLH5 expression in *E. coli* JM103 involved the use of partial XLH5 cDNA clones, isolated and identified by Rutledge *et. al.* in 1988, since these clones already contained XLH5 cDNA under transcriptional control of the *lacZ* promoter. To determine if XLH5 cDNA had any effect on *E. coli*, a series of duplicate cultures of JM103 were inoculated with bacteriophage M13mp9, XLH5A-2, XLH5A-2X, XLH5A-1 or XLH5A-1X at a multiplicity of infection (m.o.i) of 1, and growth of the cultures was monitored before and after induction of the *lacZ* promoter with 0.5 mM IPTG. Effect of XLH5 expression on bacteriophage M13mp9 was determined by measurement of phage titre of culture supernatants, at several time points.

Bacterial growth, graphed in figure 3, showed that growth of control and experimental cultures was comparable before and after induction. These observations indicate that XLH5 expression had no effect on bacterial growth. Accompanying phage titration data, also graphed in figure 3, showed that XLH5A-2, which contained the almost complete XLH5 cDNA, had a remarkably low titre prior to induction. This observation suggested that XLH5 may affect bacteriophage replication. However, when re-titration of phage stocks was carried out, as a control, it was found that XLH5A-2 had undergone degradation. After the stock was replenished, a second experimental trial followed, involving XLH5A-2 and XLH5A-2X. Observed phage titres were consistent during this trial (data not shown). Even though the data were contradictory, it remains remarkable that the phage titre of *E. coli* XLH5A-2 was able to increase over the control by a factor of almost four logarithms in the same time frame.

Since the phage titration data were contradictory, M13mp9 expression clones were sized to determine if any deletions had occurred. A deletion in the clones,

Figure 3. Determination of effect of XLH5 expression in *E. coli* using parameters of growth and phage production

Duplicate cultures of *E. coli* cells were transfected with M13mp9 and vector harbouring XLH5 with an m.o.i. of 1. At the early exponential growth phase, the cultures were exposed to IPTG at a final concentration of 0.5 mM and samples were harvested from each culture at several time points.

Panel A: JM103 transfected with M13mp9 (control).

Panel B: JM103 transfected with XLH5A-2.

Panel C: JM103 transfected with XLH5A-1X.

Panel D: Bacteria transfected with XLH5A-1.

Upper portion of each panel is a phage titration curve. Note that in the logarithmic scale for phage titre, numbers greater than 10 000 000 appear in scientific notation with a four digit mantissa and two digit exponent. The lower portion of each panel represents an average of bacterial growth, for the two cultures, over the time course of analysis.

Legend: ●, non-induced culture; +, induced culture; arrow, time of addition of 0.5 mM IPTG.

especially in the coding region for XLH5, would lead to either failure of XLH5 expression or alteration in XLH5 product which, in either case, would explain loss of biological activity. The results of the sizing experiment, shown in figure 4, revealed that XLH5A-2 was greater in size than XLH5A-2X and indicated that M13 was an unstable expression system. However, the results did not rule out the possibility that a deletion had occurred within the coding region for XLH5 in XLH5A-2. For this reason, immunological screening of plaque lifts was carried out, as described in Rutledge *et. al.* (1988), in order to determine if XLH5 expression product was present in induced transfected cells. Intense purple spots were observed on plaque lifts, which corresponded to plaques from the M13 expression clones. In contrast, signals were not observed on the plaque lifts of *E. coli* transfected with either M13mp9 or XLH5A-1X. The observation of putative expression product ruled out the possibility that a deletion had occurred that caused XLH5 not to be expressed.

These preliminary experiments indicated that apparently high-level partial XLH5 expression probably did not affect either bacterial growth or phage production. However, there were technical difficulties in carrying out the M13 mediated expression of XLH5, particularly phage instability.

2. pUC118,119 vector

a) Subcloning and expression of partial XLH5 cDNA in pUC118,119

XLH5 partial cDNA was subcloned into the pUC vector system in order to provide stabler expression than obtained with M13, used by Rutledge *et. al.*, 1988, and more importantly, to facilitate genetic engineering work for yeast expression studies. As shown in figure 5, subcloning of XLH5 cDNA involved isolation of the *HindIII-EcoRI* fragment of XLH5A-2 and directional cloning of the fragment into pUC118 and pUC119.

Expression studies were carried out with constructs p9H5A-1 and p8H5A-1,

Figure 4. Determination of partial cDNA insert sizes in M13mp9 constructs
Supernatants containing phage were lysed and electrophoresed in 0.6% agarose in 1 X TBE in the absence of ethidium bromide for 400 V h. The gels were stained in ethidium bromide solution ($5 \mu\text{g mL}^{-1}$) for 5 min and destained in 1 X TBE for 20 min.

Lanes: a, M13mp9; b, W3-29 clone; c, M13mp8; d, XLH5A-1; e, XLH5A-1X; f, XLH5A-2; g, XLH5A-2X; h, M13mp9 (90 min p.i.); j, XLH5A-2X (90 min p.i.).

a b c d e f g h i j

Figure 5. Subcloning of XLH5 in pUC118,119

An illustration of the stepwise method of subcloning of XLH5 partial cDNA into pUC118 and pUC119 phagemids. All three DNA constructs were first cut with *EcoRI* and *HindIII*. The *EcoRI-HindIII* fragment containing XLH5 cDNA was purified and ligated with either pUC118 or pUC119 to produce either p8H5A-1 or p9H5A-1.

Legend: IG, M13 intergenic region required for ssDNA replication; ORI(ColE1), dsDNA replication origin of plasmid; Ap^r, β -lactamase gene required to convey ampicillin resistance; cross-hatched zone, approximate location of *X. laevis* H5 cDNA insert. Restriction sites: H, *HindIII*; E, *EcoRI*.

in order to verify observed XLH5 expression. Similar to the previous section, growth of clones was monitored before and after 0.5 mM IPTG induction at mid-exponential growth phase. As shown in figure 6, no change in growth rate of the p9H5A-1 transformant was evident after induction, and growth curves of p9H5A-1 and p8H5A-1 transformants were comparable to that of pUC119.

Intracellular proteins of the pUC clones were subjected to SDS-PAGE, as shown in figure 7A. Two immunoblots, generated using anti-XLH5 antibody cross-reacted to two different extents with *E. coli* lysates, are shown (figure 7B, 7C and table 5). Figure 7B illustrates the *E. coli* proteins, immunologically related to XLH5, that were visualized using anti-XLH5 antibody which was weakly cross-reacted with *E. coli* intracellular proteins. This technique resulted in the detection of protein bands of M_r "34.8, 31.5, 30.5k" only in the samples obtained from transformants containing p9H5A-1. It is notable that, at this dilution, one of the molecular weight markers, bovine erythrocyte carbonic anhydrase (M_r 29 kDa), was also detected. This latter observation suggests that anti-XLH5 is not completely specific.

Conditions for immunodetection were optimized, in order to observe maximal signal to noise ratio, by observing immunoreactions carried out with several concentrations of primary antibody and adjusting secondary antibody concentration at the optimal primary antibody concentration. These optimal conditions were used on a similar electroblot, shown in panel 7C, and showed exclusive visualization of only a faint smeared band of M_r "30k" in experimental lanes (lanes b through g). This result suggests that this constitutive "30k" expression product was recognized to a greater degree than any of the other proteins. In contrast to the above, an XLH5 expression product was not observed among intracellular p8H5A-1 proteins (Fig 7D,E). Comparison of the Coomassie blue stained proteins indicated that an obvious "30k" band occurred in all preparations, but because the immunopositive protein was only among transformant proteins, it must be XLH5 related.

Figure 6. Growth curves of *E. coli* transformed with partial XLH5 cDNA (aa 10- aa 196)

Duplicate *E. coli* cultures were grown in LB medium with Ap. At the mid-exponential phase of growth, the *lacZ* promoters of the pUC vectors were induced by addition of IPTG, to a final concentration of 0.5 mM.

Panels A, B, C represent average growth of cultures of pUC119, p8H5A-1 and p9H5A-1 transformants.

Symbols: ■ , uninduced culture; + , IPTG induced culture; arrow, time of IPTG addition.

A **B** **C**

Figure 7 Analysis of intracellular proteins of *E. coli* harbouring plasmid with partial XLH5 cDNA

Total proteins were extracted from induced or uninduced transformed cells and separated in duplicate SDS-PAGE gels as described in Materials and Methods section G. The proteins of one of the gels was electroblotted onto a nitrocellulose filter and subjected to immunoblotting with anti-XLH5 antiserum coupled with biotinylated goat anti-guinea pig immunoglobulin antibody.

Panel A: Time course of p9H5A-1 transformed *E. coli* proteins fractionated on an SDS/PAGE gel

lanes: a, 5 μ g *X. laevis* LHE (donated by Dr. Neelin); b,d,f, induced p9H5A-1 (90, 60, 30 min post induction (p.i.)); c, e, g, uninduced p9H5A-1 (90, 60, 30 min p.i.); h,j, induced pUC119 (60, 30 min p.i.); i,k, uninduced pUC119 (60, 30 min p.i.); l, induced JM103 (30 min p.i.); m, uninduced JM103 (30 min p.i.); n, blank; o, low MW markers. Sample loads were approximately 10 μ g.

Panel B: Immunoblot of panel A using a 1/100 dilution of anti-XLH5 antiserum that was not cross-reacted with *E. coli* lysate. Lanes are the same as in panel A.

Panel C: Immunoblot of panel A using a 1/50 000 dilution of anti-XLH5 antiserum that was cross-reacted with *E. coli* lysate. Lanes are the same as in panel A, except for lanes a,o, which are low MW marker; n, 100 ng of *X. laevis* LHE.

Panel D: Time course of p8H5A-1 protein fractionated on SDS/PAGE

lanes: a,m, low MW marker; b,d,f, induced p8H5A-1 (90, 60, 30 min p.i.); c,e,g, uninduced p8H5A-1 (90, 60, 30 min p.i.); h,j, induced pUC119 (60, 30 min p.i.); i,k, uninduced pUC119 (60, 30 min p.i.); l, 1 μ g of *X. laevis* LHE. Sample loads were approximately 10 μ g.

Panel E: Immunoblot of panel D using a 1/100 dilution of anti-XLH5 antiserum that was not cross-reacted with *E. coli* lysate. The order of samples is the same as in panel D.

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a b c d e f g h i j k l m n o

a b c d e f g h i j k l m

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 43
 20
 16.4
 14.3

a b c d e f g h i j k l m

Abstract

Table 5. Intracellular proteins of *E. coli* strain JM103 and transformants harbouring XLH5, recognized by anti-XLH5 antiserum at two dilutions

SAMPLE	IMMUNODETECTED PROTEINS (APPARENT M_r)	
	1:100 ANTI-XLH5 ^a	1:50 000 ANTI-XLH5
JM103	35	
pUC119	35	
p9H5A-1	35, 31.5, 30.5	31 ^a
carbonic anhydrase ^b	29	-
XLH5	29.5	+
XLH1	35	+

^a = High background obscured interpretation

^b = carbonic anhydrase of bovine erythrocytes (molecular weight marker)

* = estimated size (no interpolation of molecular size could be made)

- = Negative

+ = Positive

b) Design and characterization of XLH5 clones with full XLH5 cDNA

Clones containing full length XLH5 cDNA were generated from p9H5A-1 and p8H5A-1, using synthetic oligonucleotides, following the strategy diagrammed in figure 8A. Linkers H5BP-F and H5PB-R, illustrated in figure 8B, not only reconstitute the six missing amino acids of XLH5A-2 (Rutledge *et. al.*, 1988), but also facilitate cloning and expression of XLH5. The complete XLH5 coding region can be cleaved out of the plasmids p9H5A-2, p8H5A-2 using *Bam*H1. The first ATG, putative start codon, was made to occur in a favourable context for translation initiation (ie. a purine in position -3 and a guanine in coding position 4 (Kozak, 1989)) by selection of preferred codons for *E. coli* that were compatible with *S. cerevisiae* as proposed by Bennetzen and Hall (1982). The validity of this clone was ensured by sequencing of the plasmid, as shown in figure 9.

c) Expression of full XLH5 cDNA using pUC118,119

The effect of the full length XLH5 cDNA expression plasmid in *E. coli* was assayed by inducing mid-exponential growth phase cells and monitoring their growth. As shown in figure 10, no change in the growth rate of p9H5A-2 transformant was evident after induction. Since growth of all transformants was comparable, it does not appear that XLH5 expression had an effect on *E. coli*, but it's expression was not verified by intracellular analysis of *E. coli* p9H5A-2 or p8H5A-2 proteins.

B. Expression of XLH5 in *S. cerevisiae*

The second and main approach taken to study XLH5 heterologous expression was to express XLH5 in *S. cerevisiae* and to observe growth of the transformant before and after induction.

1. YEp51 derived constructs

XLH5 was placed under transcriptional control of the inducible *GAL10* promoter using the cloning strategy illustrated in figure 11. The strategy involved

Figure 8. Reconstruction of full length XLH5 coding region by use of synthetic linkers

Panel A: Illustrates the strategy used to reconstruct the missing 9 amino acids of XLH5 cDNA using synthetic oligonucleotide linkers H5PB-F and H5PB-R. Plasmid p8H5A-2 and p9H5A-2 contain XLH5 cDNA cloned in the *Bam*H1- *Eco*R1 site of pUC118 and pUC119 respectively. Legend: IG, M13 intergenic region required for ssDNA replication; ORI(ColE1), dsDNA replication origin of plasmid; Ap^r, β -lactamase gene required to convey ampicillin resistance; cross-hatched zone, approximate location of *X. laevis* H5 cDNA insert; ATG, translational start codon of XLH5 coding sequence; TAA, stop codon of XLH5 coding sequence.

Panel B: Nucleotide sequence of linkers H5BP-F (39mer) and H5PB-R (35 mer), which were used to reconstitute the partial XLH5 cDNA. The resultant clones contain the six missing amino acids which include a translational start codon. The reconstructed XLH5 coding region can be removed from the plasmid by cleavage with the *Bam*H1 restriction endonuclease. Three-letter amino acid code is shown above the nucleotide sequence. Legend: dagger symbols= nucleotide choices favourable for translation initiation (purine at position at nucleotide position -3 and a guanine at +4 with respect to the translation start site (Kozak, 1989); outlined upper case letters= oligonucleotide; lower case letters= partial XLH5 cDNA; underline = restriction site).

A

B

Figure 9. Sequence verification of the reconstructed 5' end of XLH5

Correct incorporation of synthetic linker sequence into the 5' end was verified by sequencing of the plasmid. Panel A shows an autoradiograph of the sequence of *lacZ/XLH5* using a reverse sequencing primer (5' AACAGCTATGACCATG 3') for M13. Panel B shows the interpreted sequence with 3-letter amino acid code shown beside the sequence (Legend: lower case = portion of the multiple cloning site of the pUC vector; boxed upper case = sequence of oligo H5BP-F; upper case at 3' end = origin position of XLH5 cDNA).

A

B

ACGT

Met

Ala

Glu

Asn

Ser

Ala

Ala

Thr

Pro

Ala

← TRANSLATIONAL
START SITE

Figure 10. Growth curves of *E. coli* transformed with full XLH5 cDNA

Duplicate *E. coli* JM103 cultures were grown in LB with λ p. At early exponential phase of growth, indicated by the arrows, the cultures were induced by the addition of IPTG to a final concentration of 0.5 mM.

Panels A, B, C represent average growth of cultures of the pUC119, p8H5A-2 and p9H5A-2 transformants.

Symbols: ■ , non-induced culture; +, induced culture; arrow, time of addition of IPTG.

A

B

C

2 of/de 2

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

Figure 11. Construction of YEpH5A-i2 and YEpH5A-2

Plasmid YEpH5A-i2 contains reconstructed XLH5 cDNA inserted in the *Bam*HI site of YEp51 whereas plasmid YEpH5A-2 contains reconstituted XLH5 cDNA inserted in the *Sal*I to *Bam*HI site of YEp51. The *Sal*I-*Bam*HI ends were ligated after both ends were filled in with dNTPs and T4 DNA pol.

Legend: *GAL*10, 0.5 kb *S. cerevisiae* *GAL*10 promoter; Ap^r, *E. coli* β -lactamase gene for ampicillin resistance; ORI (2 μ), *S. cerevisiae* 2- μ m circle origin of replication; ORI (ColE1), *E. coli* ColE1 origin of replication; REP3, *S. cerevisiae* 2- μ m circle REP3 locus; t, *S. cerevisiae* 2- μ m circle termination site; cross-hatched zone, *X. laevis* H5 cDNA; jagged line, *E. coli* tetracycline resistance gene fragment. Restriction sites: B, *Bam*HI; S, *Sal*I; H, *Hind*III; E, *Eco*RI.

excision of the p9H5A-2 fragment and ligating one end to YEp51 cleaved with *Sall* and *Bam*HI. The protruding free ends of the recombinant product were supposedly 'filled-in' and then blunt end ligated. Ligation products were transformed into JM103, and clones such as YEpH5A-2 (figure 11, 12A) were identified on the basis of fragment sizes generated from plasmid digests. One clone, designated YEpH5A-i2, which was also chosen for initial expression studies in *S. cerevisiae* (figure 11), contained XLH5 cDNA cloned in the *Bam*HI site of YEp51. The consequence of this ligation is that the 275 bp portion of the *tet*^r gene of pBR322 (position 650-375), which should have been excised with *Sall*, separates the *GAL*10 promoter from the normal transcription phasing of the expression cassette.

1. Transformation

Three yeast strains were initially chosen as hosts for XLH5 expression (table 3). Strain ATCC 46004 was found not to be transformable using several transformation protocols (alkaline cation, spheroplasting). Strain 5143, a haploid strain possessing a variety of genetic markers including *leu2*, required for prototrophic selection, was found to be useful. The additional markers could be used for genetic manipulation of the yeast, if needed. Strain 5055, a diploid cell type which possessed only the *leu2* mutation, was chosen as an alternate host. All yeast strains were transformed with YEp51, YEpH5A-2 and YEpH5A-i2 using a spheroplasting method (Beggs, 1978).

a) Screening by colony hybridization and southern analysis of yeast transformants

Colony hybridization was used to screen for yeast transformants which contained XLH5 cDNA. Shown in figure 13A, is the result of a screening of a random selection of putative YEp51 and YEpH5A-i2 transformants by colony hybridization, using the p9H5A-2 *Bam*HI fragment as probe. Both filters showed

Figure 12. Sequence comparison of YEpH5A-i2 and YEpH5A-2 in the vicinity of the BamHI site of XLH5 cDNA

Plasmids YEpH5A-i2 and YEpH5A-2 were directly sequenced along the anti-sense strand in the region of the *GAL10* promoter using oligomer 5-D24R, which spans leu-33 to asp-24 of XLH5A cDNA (5' CAATATCATGTCAGAGTACTTAGGGTGGT 3'), as primer. Panel A: Autoradiograph of the sequencing gel used for YEpH5A-2. Panel B: Autoradiograph of the sequencing gel used for YEpH5A-i2. Panel C: Aligned sequence interpretations of panels A, B (Legend: outlined capital letters = *GAL10* promoter; bold faced capital letters = pBR322 sequence; capitals = H5BP-F sequence; double underline = *Hpa*I site; X, unidentified nucleotide (should be guanine)).

YEPHSA-12

B

T
G
C
A

C

3'
3'

GCXCATCTCGTAG
AACTCAGCTCTAG

GCAATGTACCGTCTCT
GCAATTGTACCGTCTCT

5'
5'

A

C
G
T
A

YEPHSA-2

Figure 13. Colony hybridization and southern analysis of putative *S. cerevisiae* 5143 YEpH5A-i2 transformants

Panel A Autoradiographs of nylon filters with grids of either YEp51 (control) or YEpH5A-i2 putative transformants. Colony hybridization was performed as described in materials and methods (section E c) using an [α 32 P] labelled 0.8 kb *Pvu* II-*Eco* RI fragment of XLH5.

Panel B Electrophoretic analysis of total *S. cerevisiae* DNA digested with *Hin* dIII in a 0.7% agarose gel. Sample loads were approximately 5 μ g.

Lanes: a, *Hin* dIII digest of lambda^{*}; b, *Hin* dIII digest of YEpH5A-i2; c, blank; d, digested non-transformed *S. cerevisiae* 5143; e-m digested putative transformants.

Panel C Southern blot of panel B probed with an [α 32 P] labelled *Pvu* II-*Eco* RI fragment of XLH5.

Panel D Electrophoretic analysis of uncut total DNA electrophoresed on a 0.7% agarose gel. Layout as in panel A with only e-n containing undigested transformant DNA. Sample loads were approximately 5 μ g.

Panel E Southern blot of panel D probed with an [α 32 P] labelled *Pvu* II-*Eco* RI fragment of XLH5.

^{*} Sizes (kb) of lambda phage DNA digested with *Hin* dIII are:

23.130, 9.416, 6.557, 4.361, 2.322, 2.027, 0.564, 0.125⁺

⁺ difficult to detect due to small size

A

YEp51

YEpH5A-12

B

a b c d e f g h i j k l m

C

a b c d e f g h i j k l m

D

a b c d e f g h i j k l m n

E

a b c d e f g h i j k l m n

signals when probed with the p9H5A-2 *Bam*HI fragment, however, the signals from the filter containing putative YEpH5A-i2 transformants were stronger.

A second and more informative proof of transformation involved southern hybridization of *Hind*III digested and uncut genomic DNA from colony hybridization positives, as shown in figures 13, 14, 15, 16 and 17. *S. cerevisiae* strains 5143, 5055 do not have any XLH5 sequences, shown by lack of hybridization of XLH5 probe to uncut and cut non-transformed genomic DNA (eg. lanes c,d in Fig. 14B and D). Since all *Hind*III genomic fragments which hybridized to XLH5 probe were of the same size as linearized vector, the data imply that the plasmid DNA, originally used to transform yeast, replicated as extrachromosomal DNA. Strain 5143 has a higher copy number of this DNA than strain 5055 (figure 14 versus figures 15-17), as shown by the increased ratio of signal intensity between linearized intracellular plasmid vector and linearized purified vector.

Figure 17 panel C shows that strain 5055 was [*cir*⁺], that is it contained endogenous 2- μ m circles, because certain genomic DNA fragments, characteristic of 2- μ m DNA, hybridized to a probe specific for the endogenous plasmid (the 2.0 kb *Eco*RI fragment of YEp51). Also, figure 17 illustrates that in a relatively long exposure the p9H5A-2 probe hybridized to YEp51 (lanes a,b), indicating that this vector has some sequences related to XLH5. So that no confusion would exist in Northern analyses, segments of YEp51 related to XLH5 were identified. *Eco*RI digests of YEp51 and YEpH5A-i2 were carried out, which cleave the constructs into three separate fragments (2.0, 2.8 and 3.3 kb).

Figure 14. Southern analysis of putative *S. cerevisiae* 5143 transformants

Panel A Electrophoretic analysis of 10 μ g uncut and *Hind*III digested total DNA from *S. cerevisiae* 5143 transformed with YEp51 electrophoresed in a 0.7% agarose gel.

Lanes: a, *Hind*III digested lambda; b, *Hind*III digested YEp51; d,e, uncut and cut non-transformed strain 5143; f,g, uncut and cut putative transformant 1; h,i, uncut and cut putative transformant 2; j,k, uncut and cut putative transformant 3; l,m, uncut and cut putative transformant 4.

Panel B Southern blot of panel A probed with an [α 32 P] labelled 1.0 kb YEp51 *Pst* I fragment

Panel C Electrophoretic analysis of 10 μ g uncut and *Hind*III digested total DNA from *S. cerevisiae* 5143 transformed with YEpH5A-2 in a 0.7% agarose gel.

Lanes: a, *Hind*III digested lambda; b, *Hind*III digested YEpH5A-2; c,d, uncut and *Hind*III digested non-transformed strain 5143; e,f, uncut and cut putative transformant 1; g,h, uncut and cut putative transformant 2; i,j, uncut and cut putative transformant 3; k,l, uncut and cut putative transformant 4; m,n, uncut and cut putative transformant 5.

Panel D Southern blot of panel C probed with an [α 32 P] labelled 0.8 kb *Bam*H1 p9H5A-2 fragment.

A

a b c d e f g h i j k l m

B

a b c d e f g h i j k l m

C

a b c d e f g h i j k l m n

D

a b c d e f g h i j k l m n

Figure 15. Southern analysis of *S. cerevisiae* 5055 transformed with YEp51

Panel A Electrophoretic analysis of uncut and *Hind*III digested *S. cerevisiae* 5055 DNA electrophoresed in a 0.7% agarose gel.

Lanes: a, *Hind*III digested lambda; b, *Hind*III digested YEp51; c, blank; d,e, uncut and cut non-transformed strain 5055; f,g, uncut and cut putative transformant 7; h,i, uncut and cut putative transformant 8; j,k, uncut and cut putative transformant 9; l,m, uncut and cut putative transformant 11; n, blank.

Panel B Southern blot of gel probed with an [α 32 P] labelled 1.4 kb *Sal*I-*Eco*RI YEp51 fragment.

Panel C Southern blot of gel probed with an [α 32 P] labelled 2.0 kb *Eco*RI YEp51 fragment

Figure 16. Southern analysis of *S. cerevisiae* 5055 transformed with YEph5A-2

Panel A Electrophoretic analysis of 10 μ g uncut and *Hind*III digested *S. cerevisiae* 5055 DNA electrophoresed in a 0.7% agarose gel.

Lanes: a, *Hind*III digested lambda; b, *Hind*III digested YEph5A-2; c, blank; d, *Hind*III digested non-transformed strain 5055 DNA; e,f, uncut and cut putative transformant 1; g,h, uncut and cut putative transformant 2; i,j, uncut and cut putative transformant 3; k,l, uncut and cut putative transformant 4.

Panel B Southern blot of panel A probed with an [α 32 P] labelled 0.8 kb p9H5A-2 *Bam*HI fragment.

A

a b c d e f g h i j k l

B

a b c d e f g h i j k l

-

-

-

-

Figure 17. Southern analysis of *S. cerevisiae* 5055 transformed with YEpH5A-i2

Panel A Electrophoretic analysis of uncut and *Hind*III digested strain 5055 DNA electrophoresed in a 0.7% agarose gel.

Lanes: a, *Hind*III digested lambda; b, *Hind*III digest YEp51; c, *Hind*III digest of YEpH5A-i2; d, blank; e,f, uncut and cut non-transformed strain 5055; g,h, uncut and cut putative transformant 6; i,j, uncut and cut putative transformant 22; k,l, uncut and cut putative transformant 24; m,n, uncut and cut putative transformant 26.

Panel B Southern blot of panel A probed with an [α 32 P] labelled 0.8 kb p9H5A-2 *Bam*HI fragment.

Panel C Southern blot of panel A probed with an [α 32 P] labelled 2.0 kb YEp51 *Eco*RI fragment.

A

abcde fghi / klmn

B

abcde fghi / klmn

C

abcde fghi / klmn

Figure 18 Southern analysis of YEp51 probed with XLH5 cDNA

Panel A Electrophoretic analysis of uncut and *EcoRI* digested YEp51 and YEpH5A-i2 in 0.7 % agarose. Lanes: a, *HindIII* digested lambda; b, uncut YEp51; c, *EcoRI* digested YEp51; d, uncut YEpH5A-i2; e, *EcoRI* digested YEpH5A-i2.

Panel B Autoradiograph of a southern blot of panel a probed with an [α 32 P] labelled p9H5A-2 *Bam*HI fragment.

A

B

Figure 18B illustrates the southern analysis of YEp51 and YEpH5A-i2 *EcoRI* fragments probed with XLH5 cDNA. After a moderate stringency wash (65 °C, 0.3M NaCl, 0.03 M sodium citrate), strong signal was observed for the 2.8 kb fragment of YEpH5A-i2 digested with *EcoRI*. Weak signal was also observed for the 2.0 and 3.3 kb *EcoRI* fragments of both YEp51 and YEpH5A-i2. As shown in the illustration of YEp51 (figure 19), these fragments contain different proportions of various yeast and bacterial sequences. Comparison of available nucleotide sequences within YEp51 and XLH5A cDNA was made using the FASTA software package of the N.R.C.C. and summarized in figure 19.

In order to ensure that point mutations did not occur within the promoter regions of the expression plasmids, recovery from yeast transformants was attempted, however, only 5143/YEpH5A-2 was recovered and sequenced. This data indicated that no point mutations occurred within the *GAL10* promoter.

2. Growth and XLH5 transcription in 5143/YEpH5A-i2

a) Glucose/ galactose carbon shift regime

Growth of transformants was monitored, during galactose induction experiments, in order to determine whether XLH5 expression had an effect on yeast cell proliferation. Induction experiments were carried out by growing transformants in minimal medium with glucose until early exponential growth phase, at which time cells were quickly harvested and resuspended in either minimal medium with glucose or galactose. As shown in figure 20A, growth of transformant 5143/YEpH5A-i2 ceased when mid-exponential phase cells were exposed to galactose as a sole carbon source. Northern blot analysis, shown in figure 20B, revealed that three size classes of constitutively expressed transcripts hybridized to XLH5: 2.3, 1.2 and 0.7 kb.

Figure 19 Positions of overlaps that exist between XLH5A cDNA and the YEp51 vector

Approximate positions of overlapping sequences are indicated by stars: a single star designates an overlap with the anti-sense strand, double stars designate an overlap with the sense strand. In brackets, below the stars, the length of overlap and the number of exact nucleotide homologies within the overlap is presented as a percentage value.

Legend: *GAL10*, 0.5 kb *S. cerevisiae* *GAL10* promoter; Ap^r , *E. coli* β -lactamase gene for ampicillin resistance; *ORI*(2 μ), *S. cerevisiae* 2- μ m circle origin of replication; *REP3*, *S. cerevisiae* 2- μ m *REP3* locus; jagged line, *E. coli* tetracycline resistance gene fragment.

b) Growth of non-transformed strain 5143

In order to determine whether failure of growth of 5143/YEpH5A-i2 in galactose was a function of XLH5 expression or because of a general metabolic deficiency, observation of parental strain growth in minimal medium supplemented with various carbon sources followed. Figure 21 illustrates that strain 5143 can metabolize glucose and sucrose but not galactose. A defect in one of the steps involved in galactose utilization could account for the lack of growth, observed in figure 20A.

c) Sucrose/galactose carbon shift

The second approach taken in determining the effect of YEpH5A-i2 directed XLH5 transcription on strain 5143 was to use sucrose, as a neutral carbon source, during the expression regime involving 5143/YEpH5A-i2. This method of induction has the advantage that a cell wash does not have to take place and galactose or glucose can be added directly to a culture of cells, although sucrose does have a minor catabolite repression effect on *GAL* genes (Hashimoto *et. al.*, 1983). However, as illustrated in figure 22A, growth of transformant 5143/YEpH5A-i2 continued and was unaffected by induction during a sucrose/galactose regime. Northern blot analysis of RNA, shown in figure 22 panels B and C, from cells exposed to either glucose or galactose revealed constitutive expression of the three classes of transcripts previously observed (refer to figure 20 B).

Figure 20. Growth curves and RNA analysis of *S. cerevisiae* 5143/YEpH5A-i2

Panel A Growth curve of transformant in minimal medium with glucose. Cultures were induced during their early exponential growth phase (see arrow) by a regime of collection, washing and resuspension in minimal medium with galactose. Total RNA was harvested from samples of approximately $0.5-1.0 \times 10^9$ cells at various time points.

Legend: • , glucose; +, galactose; arrow, time of culture resuspension in minimal medium with galactose.

Panel B Electrophoretic analysis of 5143/YEpH5A-i2 RNA (10 μ g), harvested over a time course after time of induction, in a 1.0% agarose, 0.66 M formaldehyde gel.

Panel C Autoradiograph of an electroblot of panel B probed with an [α - 32 P] labelled p9H5A-2 *Bam*HI fragment. Sizes of the three classes of transcript that hybridized to the probe were 2.3, 1.2 and 0.7 kb based on the ribosomal markers (25S = 3.4 kb and 18S = 1.6 kb according to Georgiev *et. al.*, 1981 and Rubtsov *et. al.*, 1980)

Figure 21. Growth curves of *S. cerevisiae* 5143 in various carbon sources

Growth curves of non-transformed cells grown in minimal medium with glucose to early exponential phase, indicated by the arrow. Cells to be induced were harvested, washed and resuspended in the same amount of minimal medium containing either 2% glucose, galactose or sucrose.

Symbols: arrow, time of exposure to various carbon sources; ●, glucose grown cells (not exposed to other carbon sources); +, glucose (re-exposure); □, galactose; *, sucrose.

3. Growth and XLH5 transcription in 5055/YEpH5A-i2

Because strain 5143 was defective in at least galactose utilization, and the pattern of constitutive XLH5 transcription in 5143 was not expected, the alternate host strain 5055 was chosen. With only a single *leu2*- mutation, this strain has a less mutated genome and probably, as such, an intact *GAL* regulatory apparatus.

As shown in figure 23A, growth of transformant 5055/YEpH5A-i2 continued after induction in a glucose/ galactose regime, although at a reduced rate. The stationary number of cells of the galactose induced culture was higher than the glucose grown cells and is probably the result of the increased amount of carbon source present in the culture. Northern blot analysis, shown in figure 23B, revealed the presence of three plasmid encoded XLH5 transcripts that accumulated during the observed time course. Densitometric analysis of transcript abundance, summarized in figure 24, shows that accumulation of the three transcripts over time was not equal, as the abundance of the two smaller transcripts (0.8 and 1.2 kb) increased to a greater extent with time than did the 2.1 kb size transcript.

Figure 22. Growth and RNA analysis of *S. cerevisiae* 5143/YEpH5A-i2

Panel A Growth curve of transformant in minimal medium with sucrose. Cultures were induced in early exponential phase, indicated by the arrow, by addition of galactose to 2% final concentration.

Legend: arrow, time of addition of galactose; ■, uninduced culture; +, induced culture.

Panel B Electrophoresis of 5143/YEpH5A-i2 RNA (10 µg) in a 1.0% agarose, 0.66 M formaldehyde gel.

Uninduced cells (sucrose grown) Lanes: a, 0 min; b, 30 min; c, 60 min; d, 90 min.

Induced cells (galactose grown) Lanes: e, 0 min; f, 30 min; g, 60 min; h, 90 min.

Panel C Autoradiograph of an electroblot of panel B that was probed with an [α 32 P] labelled p9H5A-2 *Bam*HI fragment.

Transcript sizes (2.0, 1.0 and 0.7 kb) were estimated from a standard curve based on electrophoretic mobilities of ribosomal markers. These transcript sizes concur with transcript size estimates for figure 20.

Figure 23. Growth and RNA analyses of *S. cerevisiae* 5055/ YEpH5A-i2

Panel A Growth curve of *S. cerevisiae* 5055/YEpH5A-i2 grown in glucose with galactose added to 2.0% when cultures were in mid-log phase of growth.

Symbols: arrow, time of addition of galactose; •, glucose grown cells; +, galactose grown cells.

Panel B Electrophoretic analysis of total RNA, 10 µg per lane, in a 1.0% agarose, 0.66 M formaldehyde gel.

Lanes: a,b, sucrose, galactose (90 min p.i.) non-transformed 5055; c,d, sucrose, galactose (90 min p.i.) YEp51 transformant.

Time course analysis of XLH5 transcripts in 5055/YEpH5A-i2 total RNA: e,f, 0 min p.i.; g,h, 30 min; i,j, 60 min; k,l, 90 min. Lanes e,g,i,k; uninduced RNA. Lanes f,h,j,l; induced RNA.

Panel C Autoradiograph of a northern electroblot of panel B that was probed with an [α 32 P] labelled 0.8 kb p9H5A-2 fragment.

Figure 24. Densitometric analysis of YEpH5A-i2 and YEpH5A-2 directed XLH5 transcription in strain 5055

Panel A A three dimensional overlapping bar chart which represents the relative transcript abundance of the three XLH5 transcripts of induced transformants, observed in the northern analysis of 5055/YEpH5A-i2 (figure 23 C), measured by a computer-scanning densitometer.

Panel B A three dimensional overlapping bar chart which represents the relative transcript abundance of the three XLH5 transcripts of uninduced transformants, observed in the northern analysis of 5055/YEpH5A-i2 (figure 23 C), measured as in panel A.

A

B

4. Growth and XLH5 transcription in 5143/YEpH5A-2

Since the main focus of the yeast heterologous expression studies was on the use of construct YEpH5A-2, most experiments carried out with YEpH5A-i2 were also carried out with YEpH5A-2.

Given the data in figures 20 and 21, showing the inability of strain 5143 to metabolize galactose, growth of 5143/YEpH5A-2 was carried out in minimal medium containing sucrose. As shown in figure 25, growth of 5143/YEpH5A-2 in YNB was identical with either sucrose or galactose and no lag in growth of induced cells was evident. Northern analysis of total RNA, shown in figures 25 B and C revealed that predominantly a single transcript of 0.9 kb in size was produced, which increased in abundance over a time course of 2.7 hours. This pattern of transcription contrasts with that observed in the northern analysis of total RNA from strain 5055 transformed with YEpH5A-i2. Another interesting observation from this experiment was that inadvertently degraded RNA from uninduced cells (lane g) still contained a moderate amount of intact XLH5 transcript, which suggests that the XLH5 specific transcript is stable relative to other yeast RNA species.

5. Growth and XLH5 transcription in 5055/YEpH5A-2

Transcription of the XLH5 gene was also observed in strain 5055 in order to confirm the regulated pattern of XLH5 transcription observed in 5143/YEpH5A-2. As plotted in figure 26A, growth of the uninduced transformant 5055/YEpH5A-2 was identical to the induced transformant after induction. Northern analysis of XLH5 transcript abundance over a six hour time period, shown in figure 26C, indicates appearance of only a 0.94 kb transcript. Some work was done to determine the time at which maximum XLH5 transcript accumulation occurred for several

Figure 25. Growth and northern analysis of XLH5 transcript produced in *S. cerevisiae* 5143/YEpH5A-2

Panel A Cells were grown in minimal medium with sucrose. When cells reached exponential phase, indicated by the arrow, cells were harvested and resuspended in 2% galactose containing medium.

Symbols: arrow, time of induction; • , uninduced culture; + , induced culture.

Panel B Electrophoretic analysis of RNA, approximately 10 μ g per lane, in 1.0% agarose, 0.66 M formaldehyde gel (0 to 60 min p.i, 30 min intervals).

Lanes: a,c,e,g, uninduced samples; b,d,f,h, induced samples. Lanes g,h, collected 2.6 hrs p.i. (Note sample in lane g appears degraded)

Panel C Northern electroblot of gel probed with an [α 32 P] labelled p9H5A-2 *Bam*HI fragment.

B

a b c d e f g h

C

a b c d e f g h

Figure 26. Growth and northern analysis of XLH5 transcript produced in *S. cerevisiae* 5055/YEpH5A-2

Panel A Growth curves of cells grown in minimal medium with sucrose and induced at mid-exponential phase, shown by the arrow, by cell harvest and resuspension in minimal medium with galactose (2% final).

Symbols: arrow, time of induction; •, uninduced culture, +, induced culture.

Panel B Time course analysis of XLH5 transcription. Electrophoretic analysis of RNA (10 μ g) in a 1.0% agarose, 0.66 M formaldehyde gel.

Lanes: a, RNA ladder; b,d,f,h, sucrose grown; c,e,g,i, galactose grown. Lanes: b,c, 0 hr; d,e, 2 hr; f,g, 4 hr; h,i, 6 hr; j, glucose (3.8 hr. p.i.); k, galactose (3.8 hr. p.i.).

Panel C Autoradiograph of an electroblot of panel B probed with an [α 32 P] labelled p9H5A-2 *Bam*HI fragment. The single transcript that hybridized to XLH5 probe is 0.94 kb in size.

time course trials. Various loads of total RNA (1 μ g, 0.5 μ g, 0.25 μ g) were slot blotted onto nylon membrane using a slot blotting apparatus and probed with 32 P labelled XLH5 cDNA. Densitometric analysis of the hybridized RNA was performed (data not shown) and indicated that maximum transcript accumulation occurred at approximately 8 hours after the time of induction.

a) Characterization of XLH5 transcript of yeast

Transcript characterization was started during expression studies involving YEpH5A-i2 in order to map the transcription initiation sites of the observed transcripts and to determine whether the transcripts were polyadenylated. Knowledge of these features, especially toward the 5' end, would allow authentication that translation likely occurred at the specified ATG start codon.

(1) Determination of polyadenylation status

Oligo(dT) cellulose chromatography of 5055/YEpH5A-i2 RNA, shown in figure 27, revealed that all three of the transcripts, that were transcribed through XLH5 cDNA, were probably polyadenylated. Despite the smeared nature of XLH5 probe hybridization to the poly(A+) lane, indicating some degradation of the RNA, the above interpretation is supported by the absence of signal in the poly(A-) lane. The migration and ability of the poly(A+) sample to hybridize to XLH5 probe may have been influenced by high salt, or SDS concentration, or impurity. Although all three of the transcripts are putatively polyadenylated, it is not known whether transcript termination is taking place within the 2- μ m-circle sequence of YEp51 or in the vicinity of the 6-mer poly(A) tail of the XLH5 cDNA.

Figure 27. Analysis of *S. cerevisiae* 5055/YEpH5A-i2 RNA fractionated on oligo(dT) cellulose

Approximately one mg of total RNA from strain 5055 transformant containing YEpH5A-i2 exposed to galactose for 30 min was applied to a 0.2 mL sterile dispocolumn (Bio-Rad) containing oligo-(dT)-cellulose washed with column loading buffer (20 mM Tris-HCl(pH7.6), 0.5 M NaCl, 1 mM EDTA (pH 8.0), 0.1% SDS). The column was washed with sterile RNase free column loading buffer containing 0.5 M NaCl to elute the poly(A-) fraction and then NaCl free elution buffer to remove the poly(A+) RNA from the oligo (dT) cellulose.

Panel A Electrophoretic analysis of RNA in 1.0 % agarose, 0.66 M formaldehyde gel.

Lanes: a, total RNA (10 μ g), b, total RNA (3 μ g); c, poly (A)⁻ fraction; d, poly (A)⁺ fraction

Panel B Northern blot probed with an [α -³²P] labelled 0.8 kb p9H5A-2 *Bam*III fragment.

A

B

a b c d

a b c d

25S —

18S —

kb

1.9

1.0

0.8

0.6

(2) 5' end determination

Primer extension analysis was carried out on total RNA from uninduced and induced cells according to the method of Calzone *et. al.*, 1987. Oligomer 5-D24R, previously used for 5' end determination of XLH5 in *X. laevis* red blood cells by Foster (1990), was also used for primer extension analysis of the yeast derived XLH5 mRNA. Since the primer spans leu-33 to asp-24 of the cDNA sequence (Rutledge *et. al.*, 1988), it was well suited for mapping the transcription initiation window in YEpH5A-2. It was not known if the primer was suitable for high resolution mapping of the 5' terminus of the YEpH5A-i2 directed XLH5 transcripts, since the primer might not be located within 200 nt of the 5' termini of the transcripts, because of the 275 bp tet^r fragment that separated the *GAL10* promoter from the XLH5 cDNA. If the transcription initiation site of the YEpH5A-i2 *GAL10* promoter is active, then a primer extension product of 330 bp should be observed; if it is not, then several transcripts with heterogeneous 5' ends would be expected.

As shown in figure 28A, primer extension with reverse transcriptase generated products of 400 and 420 nt, observed as faint bands, from the total RNA from strain 5055 transformed with YEpH5A-i2. Since the observed primer extension products were significantly larger than the expected size of the primer extension product, transcription appears to be initiated upstream, by at least 70 nt, of the site reported by Yocum and Johnston (Broach *et. al.*, 1983). Using total RNA from uninduced cells, primer extension products of 240 nt, 140 nt and smaller were observed (data not shown).

As shown in figure 28B, primer extension with reverse transcriptase mapped the major 5' end of the XLH5 transcript from strain 5143 transformed with YEpH5A-2, in the vicinity of the guanine nucleotide that follows a polyA stretch, 30 nt upstream of the first ATG (methionine codon) of the XLH5 coding sequence. This result agrees with a previous *GAL10* transcript site determination study

Figure 28. Determination of 5' end of XLH5 transcript

Panel A **Autoradiograph of oligomer 5-D24R primer extension products, generated from total RNA from 5055/YEpH5A-i2, electrophoresed in a 5% polyacrylamide gel.**

Lanes: a, [α ^{32}P] endlabelled *Hinf*I digested pBR322; b, extension product made from 40 μg total RNA from 5055 YEp51 grown in galactose containing medium (90 min); c, 40 μg total RNA from 5055/YEpH5A-i2 grown in galactose containing medium (90 min); d, [γ ^{32}P] endlabelled *Eco*RI, *Hind*III digested lambda DNA.

Panel B **Autoradiograph of end-labelled oligomer 5-D24R primer extension products, generated from total 5143/YEpH5A-2 RNA, electrophoresed in an 8% polyacrylamide sequencing gel alongside a sequencing ladder generated from YEpH5A-2 using oligo 5-D24R as primer.**

Lanes: a, oligomer 5-D24R; b, products from total RNA from 40 μg 5143/YEpH5A-2 (uninduced 30 min); c, products from 40 μg total RNA from 5143/YEpH5A-2(induced 3.8 hr(glu/gal regime)).

Legend: lower case = YEp51 promoter; upper case = dNTP fill in; boxed upper case = oligo H5PB-R; 1 = 5' end determination performed by Yocum *et. al.*, 1982 in Broach *et. al.*, 1983; 2 = 5' end determination of Johnston and Davis, 1984; * = this 5' end determination.

(Broach *et. al.*, 1983).

2. Immunodetection of XLH5 product in *S. cerevisiae*

In order to determine if XLH5 was expressed and to what extent it accumulated in *S. cerevisiae* strain 5055, transformants containing YEp51 (control), YEpH5A-2 and -i2 were grown in liquid culture to mid-log growth phase and induced. Then, over the course of several hours, aliquots of cells were removed and intracellular proteins extracted. Protein samples were fractionated in SDS/PAGE gels and electroblotted onto a nitrocellulose support. Proteins recognized by anti-XLH5 antibody were visualized by the same immunodetection regime as used for *E. coli* (Materials and Methods section G2b)

Figure 29B shows that a faint "19k" protein was recognized by anti-XLH5 antiserum among 5055/YEp51 proteins. Figure 29D shows that of the 5055/YEpH5A-2 proteins recognized by anti-XLH5, two are very prominent. Since only the "22k" protein was exclusive to 5055/YEpH5A-2, it appears to be derived from XLH5 cDNA. However, based on M_r in SDS-PAGE, the expression product is much smaller than authentic XLH5 and this indicates that the protein is either being degraded by the yeast host or translation starts at the internal methionine (aa 31) of XLH5 cDNA. The loss of a portion of the protein may explain why the expression product has no effect on cell proliferation.

Similar to the above immunodetection studies, 5055/YEpH5A-i2 proteins, isolated between 4 and 7 hours post induction, were probed with anti-XLH5 antibody and no immunoreactive bands were observed (data not shown). These results support the possibility that translation of the XLH5 transcripts, directed by YEpH5A-i2 may have occurred at an inappropriate ATG codon resulting in rapid loss or mistranslation of H5 sequence.

Figure 29. Identification of heterologous XLH5 product in *S. cerevisiae* 5055/YEpH5A-2

Total proteins were extracted from cells exposed to sucrose or galactose were separated by duplicate SDS-PAGE gels as described in Materials and Methods section G. The proteins of one of the gels were electroblotted onto a nitrocellulose filter and immunoreacted with anti-XLH5 antiserum and biotinylated goat anti-guinea pig immunoglobulin.

Panels A,B: Determination of XLH5 gene product among total proteins of induced YEp51 and YEpH5A-2 transformants.

Panel A: Coomassie stained gel containing yeast total protein extracts, approximately 15 μ g per lane. Lanes: M, molecular weight marker; a, 3 μ g *X. laevis* LHE (linker histone extract); b, non-induced 5055/YEp51; c, 5055/YEp51 (4 hrs p.i.); lanes d-i, total protein extracts from non-induced and induced 5055/YEpH5A-2 transformants at 2, 4, 6 hrs p.i, arranged in alternating order.

Panel B: Immunoblot of panel A using a 1: 50 000 dilution of anti-XLH5 antiserum that was not cross-reacted with yeast lysate. Lane a contained 50 ng of LHE.

Panels C,D: Time course determination of XLH5 gene product among total proteins of induced 5055/YEpH5A-2.

Panel C: Coomassie stained gel containing yeast total protein extracts, approximately 15 μ g per lane. Lanes: M, molecular weight markers; a, 3 μ g *X. laevis* LHE (linker histone extract); b-g, total protein extracts from induced 5055/YEpH5A-2 cells at 4,6,7,8,9,10 hr p.i.

Panel D: Immunoblot of panel C using a 1: 50 000 dilution of anti-XLH5 antiserum that was cross-reacted with yeast lysate. Samples were applied in the same order as panel C, except that 50 ng LHE was used in lane a.

IV. DISCUSSION

A. Expression of XLH5 in *E. coli*

1. Experimental design

For this thesis, *E. coli*, which has proven to be an important model for studying the expression of a number of eukaryotic proteins, was chosen as a host for XLH5 expression. As mentioned in the introduction, the *E. coli* nucleoid is similar to that of the eukaryotic chromosome in that it has similar-sized domain loops and nucleosome-like structures. Also, because many essential *E. coli* genes are available during the vegetative growth phase, expression of XLH5, if deleterious, might be easily detected by a simple change in growth rate. The use of a regulatable promoter, such as *lacZ*, potentially further allows expression of XLH5 at any stage of host cell growth. However, as discussed below (section A2), none of the experiments permitted any rigorous interpretation.

Experimental controls for this study involved the demonstration that no XLH5 expression products existed in either non-transformed *E. coli*, or were expressed from a pUC119 transformant. Immunological analysis using a high concentration of non-competed anti-XLH5 antiserum on total proteins of *E. coli* (diluted 1:100) showed that only an immunoreactive protein with an apparent M_r of 35 kDa was detected among intracellular proteins of JM103 and those of a pUC119 transformant. This protein was not detected using a lower concentration of anti-XLH5 antiserum (1: 50 000) that was also preabsorbed with total intracellular proteins from a pUC119 transformant. Purification of the immunoreactive protein and determination of its identity by microsequencing would be interesting to carry out in order to determine whether this protein is related to one of the histone-like proteins of *E. coli* (refer to Introduction).

2. XLH5 expression

The data obtained for XLH5 expression in *E. coli* shown in figures 3,6 and 10 indicate that no significant change in growth rate occurred when either M13 phage or pUC derivatives were used as vectors for expression of XLH5. One interesting observation was the inhibitory effect on production of M13 phage containing the nearly full-length XLH5 cDNA (containing amino acids 10 to 196) in one trial (figure 3). However, this observation was not seen in subsequent trials. The increase in recombinant phage production in mid to late-log cultures (after 6 hours), shown in figure 3, may be related to the known increase in proteolysis (Lo *et. al.*, 1988) which would prevent XLH5 accumulation.

Both phage-infected and plasmid-transformed *E. coli* carrying the *XLH5/lacZ* fusion were found to react positively with the anti-XLH5 antiserum, which was expected, based on earlier work by Rutledge *et. al.*, 1988. The immunoblot analysis of total intracellular polypeptides harvested from various transformants, involving an optimized immunodetection regime (figure 7C), revealed one faint "30k" polypeptide in samples from either induced or non-induced cells; this was slightly larger in size than native XLH5 (M.W. 21 kDa) and close to the size estimated for a *lacZ*-H5 fusion product based on codons from the gene sequence.

Despite the problem of "leaky" or permissive expression by the *lacZ* promoter, which has been observed by other researchers, this promoter has been used to initiate transcription of genes of different sizes such as endocellulases, growth hormone, hepatitis B antigen, human insulin, interferon, parathyroid hormone, and viral capsid protein (Baird *et. al.*, 1990 ; Charney, 1980; Goeddel *et. al.*, 1979; Lo *et. al.*, 1988 ; Tanaka *et. al.*, 1983; Tajbakhsh *et. al.*, 1990).

Regrettably, the antiserum used to study H5 expression was not very specific (Shay *et. al.*, 1988). At a high concentration, this antiserum cross-reacted with bovine erythrocyte carbonic anhydrase (M_r 29 kDa), which is one of the standard molecular

weight markers used in this work (figure 7B). This protein is a small zinc metalloenzyme which has wide tissue distribution and is very high in concentration in erythrocytes (reviewed in Davis, 1961). It is possible that the original XLH5 fraction, used as immunogen (see Appendix 4), also contained some of this enzyme. However, comparison of the amino acid sequence corresponding to XLH5 with the available carbonic anhydrase sequences revealed negligible alignment. Also, this antiserum recognized at least two other proteins: a "35k" protein in *E. coli* and a "19k" protein in *S. cerevisiae* (described in section B3) which makes it difficult to use in detailed critical studies correlating biological activity with XLH5 abundance.

One of the other weaknesses of the *E. coli* work was that XLH5 expression was not tested using the pUC plasmid with the reconstituted XLH5 cDNA (p9H5A-2). Such a test is especially important, given that to date, an entire LH gene has not been expressed in *E. coli*. When Graziano *et. al.* (1990) engineered expression of a gene coding for the chicken H5 globular fragment (264 bp), which lacked the basic C-terminus that is believed to bind to DNA, no unusual effects on *E. coli* growth were observed. No unusual effects on *E. coli* growth were observed when Smith *et. al.* (1988) expressed cDNA encoding the B4 protein of *X. laevis*. This is a 29 kDa protein that is believed to be H1-like in structure because of a predicted tripartite conformation which consists of a hydrophobic central domain flanked by an amino-terminal segment and a long carboxy-terminal tail with a tandemly repeated amino acid motif.

B. Expression of XLH5 in yeast

1. Experimental design

Expression of XLH5 was carried out in the lower eukaryote *S. cerevisiae* because this organism has chromatin and nucleosome structures, which are very similar to those of a higher eukaryote, but without any linker histone. This fact is

demonstrated by the lack of observation of a LH protein of known size and charge, by the relatively short nucleosomal repeat length and by the uniform sensitivity of the nucleosomes to DNase (refer to Introduction). *S. cerevisiae* was also promising to use because of the availability of known regulatory controls such as the *GAL10* promoter, which belongs to the uridine diphosphoglucose 4-epimerase gene (EC 5.1.3.2). This enzyme functions in the galactose metabolic pathway detailed in appendix 1. *GAL* promoters are relatively strong and direct transcription of between 0.25 to 1% total poly (A)+ mRNA (St. John and Davis, 1981). Also, since the *GAL* promoters are inducible, they allow the controlled high level transcription of a gene which is useful for expression of a potentially deleterious product.

Experimental controls for this expression study involved the use of yeast that were not transformed with XLH5. The control experiments of this study involved demonstration of absence of an XLH5-like gene and its expression products in *S. cerevisiae*. The southern hybridizations (figures 13 to 17) indicated that no XLH5-like sequences were present within the genomes of the yeast strains used for this study.

Weak hybridization was observed between YEp51 and XLH5 cDNA, as shown in figure 18. This hybridization may be due to the overlapping sequences identified using the FASTA software package and summarized in figure 19. Another control experiment was to demonstrate that an XLH5-like transcript was absent in either non-transformed yeast, or a yeast transformant harbouring YEp51. Figure 23 shows that no XLH5-like transcripts were detected in non-transformed strain 5055. However, the XLH5 probe did hybridize to a 2.1 kb transcript in 5055/YEp51. This transcript was probably plasmid encoded and contains some of the sequence identified from the alignments already mentioned.

A probe of a total protein extract of non-transformed yeast strain 5055, using the supplied anti-XLH5 antiserum, revealed no immunoreactive bands that would

indicate presence of an intracellular XLH5-like protein (data not shown). However, shown in figure 30, a protein with an apparent molecular weight of 19 kDa was recognized weakly by anti-XLH5 among intracellular proteins of 5055/YEp51. The identity of this protein, which is probably plasmid encoded, is unknown. Comparison of the amino acid sequence corresponding to XLH5 with the sequence corresponding to the *LEU2* gene of *S. cerevisiae*, which is encoded by YEp51, with the FASTA software, revealed negligible alignment.

2. Cloning of XLH5 cDNA in YEp51

The two constructs made in this thesis, which contain XLH5 cDNA in YEp51, were derived from the one cloning experiment. These constructs, designated YEpH5A-i2 and YEpH5A-2, are described in Table 4.

Copy numbers of both YEpH5A-2 and YEpH5A-i2 plasmids were higher in haploid strain 5143 than in diploid strain 5055. YEpH5A-2 was derived from YEp51, which was designed around sequences derived from the naturally occurring yeast 2- μ m plasmid. This is a dsDNA plasmid that is 6.3 kb in size and present at 60-100 copies per cell in most *S. cerevisiae* strains, including those used in this study (Clark-Walker and Miklos, 1974; Hartley and Donelson, 1980; Seligy *et. al.*, 1980). Hybrid plasmids, engineered from 2- μ m sequences, such as YEp51, are present in yeast at approximately 30-50 copies/cell (Gerbaud and Guerineau, 1980; Jayaram *et. al.*, 1983). Replication of yeast episomal plasmids at high copy number requires presence of *REP1* and *REP2* gene products encoded by the plasmid and a cis-active site, other than the origin, that has been designated *REP3*. Differences in copy number between strains is believed to be a function of the inhibitory effect of plasmid replication on the mitotic growth rate of the two strains.

3. Regulated transcription of XLH5 using the GAL10 promoter

The remainder of this discussion concerns the observations from experiments

involving construct YEpH5A-2, which contains the complete coding region of XLH5 cDNA in the same orientation as the *GAL10* promoter of YEp51. Discussion of the expression analysis from YEpH5A-2 pertains to both yeast promoter structure and transcriptional control and is presented in appendix 3.

The induction experiments presented in figures 20,22,23,25 and 26 were carried out by growing transformants in minimal medium containing glucose or sucrose, which do not induce the *GAL10* promoter. At the time of induction, cells were harvested and then resuspended in either galactose or non-inducing medium. Changes in cell number and RNA were monitored. Northern analysis was used to demonstrate that XLH5 transcription was controlled by the carbon source provided to strain 5143 and 5055 transformants harbouring YEpH5A-2. Proof that transcription of XLH5 was under stringent control of carbon source was demonstrated by the appearance of H5-specific mRNA when cells were exposed to galactose and not to glucose (figure 23,27). The transcript analysis of transformant 5143/YEpH5A-2 total RNA indicated that XLH5 transcript increased in abundance with time, when transformed yeast cells were incubated in galactose-containing medium. Time course analysis of XLH5 transcript produced in 5143/YEpH5A-2 using a sucrose/galactose growth regime, showed maximal abundance at 4 hr (figure 26 C), which was greater than in the glucose/galactose regime at this time point (figure 25 B,C). The lower transcript abundance of the latter regime may be a consequence of catabolite repression exerted by residual glucose in the galactose-containing medium. The observed pattern of XLH5 transcript accumulation compares favourably with the observation that *S. cerevisiae* cells in early stationary phase produced the highest amounts of bacterial β - galactosidase using a hybrid *GAL10-CYC1* promoter (Lotti *et. al.*, 1988).

The single observation that XLH5 transcript was present in a total RNA preparation containing completely degraded rRNA (figure 25 B,C) suggests that

the heterologous XLH5 transcript may be quite stable. This suggestion is consistent with the observations based on the stability of native XLH5 transcript in *X. laevis* erythrocytes (Foster, 1990).

The initiation site for the XLH5 transcript directed by YEpH5A-2 mapped in the same region as reported by Yocum and Johnston (see unpublished data in Broach *et. al.*, 1983). Although transcription initiation directed by the *GAL10* promoter of YEpH5A-2 appeared accurate, the site of transcription termination is unknown. However, the oligo-dT cellulose-bound RNA from yeast carrying the YEpH5A-i2 plasmid was enriched approximately elevenfold in the 0.8 kb *XLH5* transcripts, which indicates that the H5 mRNA was probably polyadenylated. Judging from the estimated 0.9 kb transcript size, the site of polyadenylation is probably within the 3'UTR of XLH5 cDNA and not in the 2- μ m sequence of the YEp51 vector.

3. XLH5 Expression

About 4 hours after yeast transformants harbouring XLH5 cDNA were exposed to galactose, an intracellular plasmid-encoded "22k" protein, which was recognized by anti-XLH5 antiserum, began to accumulate (figure 29). During the later stages of yeast growth, yeast metabolism slows down and catabolic activity drops, which may account for the appearance of H5 product.

Two methods were used to estimate the amount of XLH5- derived expression product in *S. cerevisiae* (Appendix 2, method A). The first estimate of the amount of XLH5 expression product was based mostly on the immunoblot containing the 8 hr post induction sample (figure 29C, lane e) and partly on the Coomassie stained gel (figure 29B). This value corresponded to 1 molecule per 2333 bp DNA or, one copy of H5 per 14 nucleosomes. The second estimate of the amount of XLH5 expression product was based solely on the densitometric analysis of the Coomassie stained gel. Densitometric scanning was carried out on both the

entire lane of the 8 hr post induction sample (figure 29D, lane e) as well as only on the protein band which corresponded to the immunologically reactive M_r "22k" protein, which was identified by electrophoretic mobility. This analysis showed that the number of molecules per bp of nuclear DNA was approximately 1 per 200, or approximately 1 per nucleosome. Of these two estimates, the former is probably more accurate because it is based on the specific abundance of XLH5, whereas in the case of densitometry of the SDS/PAGE gel, the abundance of XLH5 product would be an overestimate due to overlap with other proteins which had electrophoretic mobilities similar to XLH5. However, strictly concerning abundance, intracellular quantity of XLH5 expression product was ample enough that about 1/10 of the yeast nucleosomes could be associated with the product.

The accumulation of the immunoreactive protein was shown to have no consequence on yeast cell proliferation, although only a small amount of growth was expected. Since the heterologous protein, at an apparent M_r of 22 kDa is 27% smaller than native XLH5, proteolysis of putative XLH5 expression product may have occurred. Anti-XLH5 antiserum is known to cross-react equally well with XLH5 consisting of amino acids 11-108 as 8-196 (Rutledge *et. al.*, 1988). Since amino acids 109 through 196 are believed to be responsible for chromatin condensation (see Introduction B 2 e), a possible deletion of these sequences could explain why XLH5 has no effect on yeast growth.

C. Conclusions and Perspectives

During the course of this study, there have been three reports on avian H5 gene expression in animal cells, which contain a native H1. The approaches used in these studies are summarized below along with those of this thesis.

As reported in an abstract by Grun *et. al.* (1988), duck histone H5 under transcriptional control of the thymidine kinase promoter of the Vaccinia virus was expressed with little discernible effect on either chick embryo fibroblasts (CEF) or

viral expression. However, hormone-inducible avian H5 expression in transfected rat sarcoma cells was shown to have a significant effect on cell proliferation (Sun *et al.*, 1989). Treatment of cells with dexamethasone, a glucocorticoid, resulted in maximal nuclear accumulation of H5 after 35-40 hr with the consequence that the overall linker histone content became 1.7 times higher than in non-induced cells - a level similar to that of mature chicken erythrocytes. Conversely, the amount of native H1 decreased to approximately 0.7 times the level of non-induced cells. This observation suggested that newly synthesized H5 and H1 histones compete for binding to chromatin in this system. H5 expression was also found to be associated with changes in expression levels of different genes, although it was not clear whether reduction of specific transcription was the cause or the effect of reduced cell proliferation. Reduction of cell proliferation and altered gene expression appeared to be reversible since a reduction in H5 content was linked to a decrease in the doubling times of cells.

In a follow-up paper, Sun *et al.* (1990) measured the effect of H5 on primary and higher order chromatin structure of the transfected rat sarcoma cells. They found that H5 did not change the nucleosome repeat length, suggesting that the ratios of H5/H1 do not determine DNA repeat length in nucleosomes. They also examined the effect of H5 on higher order chromatin structure by MNase digestion of chromatin of cells, grown in the presence or absence of dexamethasone, and fractionation of digests by zonal centrifugation. Their data indicated that H5 associated chromatin was more compact than H1-associated chromatin for fractions having the same sedimentation coefficient. The ability of H5 to produce a more compact chromatin fibre was further demonstrated using H1/H5 depleted mature erythrocyte chromatin reconstituted with either H5 or H1. When H5 was used, chromatin had a higher sedimentation coefficient value than with H1. The authors believed that increased compaction or folding of H5-containing chromatin may have

contributed to the observed decreases in rates of both DNA synthesis and cell proliferation in both transfected sarcoma cells and maturing erythrocytes.

Similar to the above studies, the aim of this thesis was to test the effect of linker histone expression, but in cells which possess chromosomes that lack conventional linker histones. XLH5 appears to have no deleterious effect on either prokaryotic gene expression or chromosome packing. The reason for the lack of effect of H5 on *E. coli* expression remains unclear; it may be that prokaryotic chromatin lacks specific heterotypic protein binding sites, such as those found in eukaryotes for H5 interactions or that not enough intact H5 was synthesized. The data presented here indicate that the level of H5 expression in *E. coli* was low and unstable using the present expression system.

The studies carried out using a yeast host were more extensive than that using *E. coli* and proved to be successful in demonstrating regulated transcription of XLH5 cDNA and the expression of a product that was recognized by anti-XLH5 antiserum. Here it was likely that the "22k" H5-related product was degraded, and accumulated only after cells had completed most of their cell cycle. Several experiments need to be carried out to complete the characterization of this expression product. They include: isolation of the immunoreactive polypeptide and determination of its sequence identity in relation to native XLH5, determination of the intracellular distribution of this product and determination of whether this protein is chromatin-associated.

Appendix 1. Galactose enzyme induction in yeast

The following diagram, a modification of the original by Darnell (1986), summarizes induction of galactose enzymes in yeast. Extracellular galactose is transported into the cell by galactose permease encoded by the *GAL2* (2) gene. Galactose is converted to glucose-1-phosphate (G1P) by the enzymes galactokinase (1), α -D-galactose-1-phosphate uridylyltransferase (7) and uridine diphosphoglucose 4- epimerase (10), which are encoded by the following genes respectively: *GAL1*, *GAL7* and *GAL10*. The product, G1P, is then converted to glucose-6-phosphate by an isozyme of phosphoglucomutase and it enters the glycolytic pathway.

Expression of the galactose gene cluster on chromosome II is controlled by two unlinked regulatory loci: *GAL80* and *GAL4*. In medium without galactose, *GAL4* binds with *GAL80* to sites upstream of each *GAL* gene, but is unable to activate transcription. In the presence of galactose, *GAL80*-*GAL4* association is prevented and *GAL4* is allowed to interact with a TATA box factor. Glucose repression is a second scheme which accounts for inactivation of *GAL* promoters and enables yeasts that are growing in several carbon sources to preferentially select glucose. Although the mechanism by which glucose causes repression of *GAL* and other genes is not fully known, the coordinate regulation of several genes appears to be involved in this repression system (Flick and Johnston, 1990). Glucose appears to act on at least three separate steps on the *GAL* gene regulatory circuit: directly on promoters, on inducer levels and on *GAL4* protein (reviewed in Johnston, 1987; Flick and Johnston, 1990).

Appendix 2. Calculation of the amount of XLH5 expression product per bp of diploid *S. cerevisiae* nuclear DNA at 8 hrs post induction

Method A: Estimation based on densitometry of the Coomassie stained gel and immunoblot (figure 29 C,D)

- a) Determination of amount of XLH5 in 50 ng LHE by densitometry:
 $= \text{XLH5 signal} / \text{LHE signal} \times 50 \text{ ng}$
 $= (241.8/745.8) \times 50 \text{ ng} = 16.2 \text{ ng}$
- b) Measurement of XLH5 expression product in relation to XLH5 quantity in 50 ng LHE from (a):
 $= \text{experimental signal} / \text{XLH5 signal} \times 16.2 \text{ ng}$
 $= (1570/257.6) \times 16.2 \text{ ng} = 99 \text{ ng}/10 \mu\text{L}$
 Recall that the sample was made to 1 X laemmli buffer.
 Therefore, there are 99 ng expression product per 5 μL lysate
- c) Total XLH5 protein harvest:
 $99 \text{ ng}/5 \mu\text{L} \times 2000 \mu\text{L lysate} = 39600 \text{ ng} = 40 \mu\text{g}$
- d) Determination of number of molecules in 40 μg of XLH5A
 (From Table 1, 21 019 Da):
 $21\,019 \text{ g/mol}$
 $= 21.0 \mu\text{g/nmol}$
 $21.0 \mu\text{g}$ corresponds to 6.02×10^{14} molecules
 so $40 \mu\text{g}$ corresponds to $1.9(6.02 \times 10^{14}) = 1.2 \times 10^{15}$ molecules
- e) Total number of cells at 8 hrs post induction:
 From standard curve $\text{OD}_{600\text{nm}}$ 0.664 corresponds to $1 \times 10^9 \text{ cells mL}^{-1}$
 For 1 L there are 1×10^{12} cells
 Recall for induction a 10 fold concentration occurred; therefore in a 10 mL sample there are approx. 1×10^{11} cells
- f) Calculation of number of molecules of expression product per cell:
 $1.2 \times 10^{15} / (1 \times 10^{11}) = 12\,000 \text{ molecules per cell}$
- g) Calculation of number of molecules of expression product per bp of nuclear DNA:
 Recall that strain 5055 is a diploid.
 $12\,000 / (28 \times 10^6 \text{ bp}) = 1 \text{ molecule per } 2333 \text{ bp}$

Appendix 2. continued**Method B: Estimation based on densitometric analysis of Coomassie stained gel and Bio-rad protein assay**

a) Protein concentration of GAL8 sample as interpolated from a standard curve:

$$\begin{aligned} &= 29 \times 10 \mu\text{g total} \\ &= 290 \mu\text{g} / 100 \mu\text{L} \\ &= 2.9 \mu\text{g} / \mu\text{L} \end{aligned}$$

b) Amount of protein applied to the gel:

Recall that the sample load was $10 \mu\text{L}$ but $1/2$ of sample was $1 \times$ Laemmli buffer, therefore: $5 \times 2.9 \mu\text{g} \mu\text{L}^{-1} = 14.5 \mu\text{g}$

c) Percentage which "22k" protein comprises of total protein based on densitometry and calculated total amount:

$$\text{Percentage XLH5: } 90 / 1139 = 7.9\%.$$

$$\text{Therefore, } 14.5 \mu\text{g} (7.9\%) = 1145 \text{ ng.}$$

d) Calculated total XLH5 harvest:

$$= 1145 \text{ ng} / 5 \mu\text{L} \times 2000 \mu\text{L} = 458\,200 \text{ ng} = 458 \mu\text{g}$$

e) Determination of number of molecules in $458 \mu\text{g}$ of XLH5A (21 019 Da)

$$22(6.02 \times 10^{14}) = 1.3 \times 10^{16} \text{ molecules} \quad \text{refer to Method A part d)}$$

f) Calculation of number of expression product molecules per cell

$$1.3 \times 10^{16} / 1 \times 10^{11} = 130\,000$$

g) Calculation of number of molecules per bp of nuclear DNA

$$130\,000 / 28 \times 10^6 \text{ bp} = \text{approximately } 1 \text{ molecule per } 200 \text{ bp}$$

Appendix 3. Cryptic UAS influence on the *S. cerevisiae* GAL10 promoter

YEpH5A-i2 and YEpH5A-2 were both derived from one cloning experiment; however, expression analysis of the transformants indicated differences which are worthy of mention here. YEpH5A-i2 contains XLH5 cDNA in the *Bam*HI site of YEp51 (refer to figure 11). The consequence of the insertion into the *Bam*HI site was that a 275 bp fragment of the *tet^r* gene was placed upstream of *XLH5*. The derivation of two constructs from the same cloning experiment indicates that the cut vector used in the ligation was probably a heterogeneous mixture of *Sal*I, *Bam*HI and *Bam*HI fragments.

The expression of *XLH5* directed by YEpH5A-i2 provided useful information

for this expression study. In all YEp51 derived constructs, transcription initiates within the 500 bp promoter fragment and translation occurs from the first ATG at the 5' end of the inserted sequence (Broach *et. al.*, 1983). Since the *tet^r* fragment contains several ATG codons (at positions 394, 406, 451, 489, 553, 569, 638) and stop codons, translation could be initiated at several sites. Therefore, YEpH5A-i2 directed XLH5 expression provided an opportunity to determine the consequence of translation of a chimeric transcript composed of *tet^r* and XLH5 sequences.

The *GAL* promoter of construct YEpH5A-i2 directed transcription of multiple transcripts, that hybridized to XLH5 probe, in both haploid strain 5143 (*MAT_a*) and diploid strain 5055 (*MAT a/a*) (figures 20,22,23). The smaller two YEpH5A-i2 transcripts (1.2 and 0.8 kb) appear to be specific for YEpH5A-i2 because they were not observed in total RNA from induced 5055/YEp51 cells. The 1.2 kb transcript is within experimental error for the predicted size of transcript that includes both the 275 nt *tet^r* sequence and the coding portion of XLH5 whereas the 0.8 kb transcript is consistent in size with a transcript that was initiated within 100 nt of the translation start site of XLH5 cDNA.

Primer extension data (figure 28A) suggest that none of the *XLH5* transcripts were initiated within the *tet^r* fragment that precedes the XLH5 cDNA but rather initiation took place at approximately -33 and -53 with respect to the *GAL10* transcript initiation site (Broach *et. al.*, 1983). Despite the fact that the data may be rough because a relatively large distance separates the primer from the extension products, the data is consistent with the observed 1.2 kb transcript size. It is not known why a primer extension product of approximately 75 nt, which would correspond to the 0.8 kb transcript, was not observed.

Anti-XLH5 antiserum did not recognize a protein among total extracts of 5055/YEpH5A-i2 cells exposed to galactose over a time course of four to seven hours (data not shown). The absence of an immunoreactive protein indicates that

translation of the transcripts occurred at ATG codons that were in the wrong reading frame for the XLH5 cDNA (positions 406, 489 etc).

The most striking feature of YEpH5A-i2 directed XLH5 transcription was in the observation of patterns of expression that varied with host yeast cell type. Expression of the transcripts was completely deregulated in haploid strain 5143, as shown by XLH5 transcript presence in glucose grown cells and lack of increase of XLH5 transcript abundance when cells were exposed to galactose (figures 20,22). Unexpectedly, YEpH5A-i2 directed transcription was regulated, although incompletely, in diploid strain 5055 (figure 23). Here, the smaller transcripts (0.8 and 1.2 kb) clearly increased in abundance with time after cells were exposed to galactose. However, regulation was not complete because the three transcripts were also present during conditions of glucose growth and they also showed a slight increase in abundance with time (figure 24).

A literature search indicated that, similar to the observations of this thesis, the *Bam*HI to *Ava*I fragment of the *tet*^r gene has been shown to be capable of transcription activation. Within this fragment the sequence 5'TAGGCGCCAG3' (figure 30, position D) was identified as a probable UAS by searching for homology between the pBR322 and sequences upstream of the yeast arginase (*Car1*) gene (Sumrada and Cooper, 1985). In a more recent study by Sidhu and Bollon (1990), UAS sequences, within the *Bam*HI to *Sal*I *tet*^r fragment, were identified when it was observed that repressible acid phosphatase, from the *S. cerevisiae* *Pho5* gene, cloned in a promoter-less yeast shuttle vector, became constitutively expressed. Three specific pBR322 fragments with UASs were delineated by replacement of reporter gene UASs with pBR322 fragments and transcription factor binding sites were identified within these fragments by searching for common sequences. In such a manner, two sequences were identified within the 275 bp *tet*^r fragment, that were similar to the mammalian transcription factor (ATF) binding sequence (figure 30,

positions C). Use of synthetic oligonucleotide fragments corresponding to these consensus sequences enabled demonstration that maximum acid phosphatase gene expression was a function of both sequences.

The mechanism by which specific pBR322 sequences generate constitutive transcription in yeast is unknown and would be interesting to resolve given speculation on how UASs are able to activate transcription over distances of up to hundreds of nucleotides. Presumably, promoter augmentation with additional UAS sites at certain positions upstream of the TATA box may enable binding of activator proteins that activate RNA pol II, even in metabolic conditions that should lead to gene repression. Two models have been prepared to account for the mechanism of UAS function (Guarente, 1988; Struhl, 1989). One of these models involves contact between the activation region of an activator with a component of the transcriptional apparatus, such as RNA polymerase, along with contact to a TATA box factor. An alternate, less favoured, hypothesis for transcriptional activation involves activator function by causing local change in chromatin structure that makes the TATA box region accessible to its binding factor. Activator acidic regions could interact with histones and cause an alteration in the packing of DNA into nucleosomes.

All of the above mentioned UASs are not believed to play a major role in deregulation of the *GAL10* promoter because if promoter augmentation with these sites alone accounted for deregulation, then the same deregulated pattern of transcription should be observed in the two yeast cell types. An explanation that would more adequately explain varied deregulation would involve the presence of an UAS element which confers gene control in a specific yeast cell type. A 44 bp portion of the *tet^r* gene (figure 30, position B), that was identified by its homology to a 26 bp sequence necessary for α -specific gene expression, found in the upstream region of α -specific genes (Jarvis *et. al.*, 1988; Bender and Sprague, 1987), may fulfil

the role of a cell specific UAS, even though the element probably has no consequence for tet^F function because it occurs in the anti-sense orientation.

Explanation of how this segment is believed to function and contribute to deregulation of *GAL10* directed transcription requires an overview of how cell specialization occurs in yeast. The host strains used in this study represent two specialized cell types: haploid and diploid. Haploid cells (a or α) are specialized for mating and produce cell-specific proteins such as those needed for signalling and preparation for mating. The result of mated haploid cells is an a/α diploid cell which can undergo meiosis and sporulation. Differences in each cell type are determined by combinations of the three regulatory proteins specified by the *MAT* locus and a common protein present in all cell types designated MCM1 (reviewed in Herskowitz, 1989).

When a cell possesses the $\text{MAT}\alpha$ allele, α -specific genes are expressed but not a -specific genes. $\text{MAT}\alpha$ contains two genes that code for regulatory proteins, $\alpha 1$ and $\alpha 2$, which are expressed in α cells. $\alpha 1$ is a positive regulator for transcription of α -specific genes. The protein binds to a control region known as Q in the upstream region of an α -specific gene in concert with binding of a protein MCM1 to a P site. Although neither MCM1 or $\alpha 1$ can bind alone (Bender and Sprague, 1987), it is the MCM1 protein that is actually responsible for transcription activation. Also in α cells, $\alpha 2$ represses transcription of a specific genes by binding to control sites located within upstream regions for a specific genes, and preventing MCM1 interaction with transcriptional machinery.

Figure 30 The promoter region of YEpH5A-2 and YEpH5A-i2

Panel A shows a schematic representation of the promoter structures of YEpH5A-2 and YEpH5A-i2. Panel B shows the positions of UAS sites (A,B,C,D) referred to in the Discussion and their sequences. Note that numbers above the sequence elements B,C,D refer to positions within the pBR322 fragment. Also, within the putative UAS element, sequence matches with the consensus P,Q box are underlined.

Legend: straight line, *GAL10* promoter; cross-hatched line, XLH5 cDNA; jagged line, 275 bp *Sall-BamHI* tet^r fragment of pBR322; A, consensus GAL4 binding sites; TATA, TATA box; open box/wavy arrow, transcription initiation site for *GAL10*; B, putative 44 bp UAS (this study); C, putative UASs (Sidhu and Bollon, 1990); D, putative UAS (Sumrada and Cooper, 1985).

On the other hand, haploid cells bearing the MATa allele synthesize only protein a1 which does not play a role in programming the a-phenotype. Rather, a-specific genes are expressed because negative regulator a2 is absent and a-specific genes are not expressed because of the absence of positive regulator a1. However, the a1 protein plays a role in programming the diploid a/a phenotype, through its association with a2 to generate a novel regulatory protein that turns off expression of a large group of haploid specific genes. Haploid a/a cells also contain unassociated a2 that, in conjunction with MCM1, turns off expression of a-specific genes.

It is proposed that the putative UAS probably can only completely deregulate *GAL10* transcription in haploid strain 5143 because this cell type contains regulatory protein a1, which binds synergistically with MCM1 protein to the UAS, which in turn positively regulates transcription of *XLH5* even when cells are exposed to glucose.

In order for this scheme of *GAL10* deregulation to be feasible, it would have to be assumed that the putative 44 bp UAS is functional despite separation of the normally contiguous P and Q boxes by an 18 bp stretch as well as some sequence divergence from the consensus sequence. Otherwise, the six innermost positions of the P element, CCTAAT, which are believed to be important for MCM1 binding (Passmore *et al.*, 1989), are present in the putative UAS. Perhaps this configuration of binding sites may allow for limited but independent binding of MCM1 with the consequence of transcriptional activation under normal conditions of glucose repression. The second assumption involves accepting functionality of UAS elements, when located downstream of the mRNA initiation site, which is a property contrary to that observed in two studies of yeast promoters. It has been demonstrated that UASs of the yeast *CYC1* gene (iso 1-cytochrome c) were able to function when inverted with respect to the TATA box, but not when the UASs were

positioned downstream of the TATA box (Guarente and Hoar, 1984). In another study, the *GAL10* promoter (365 bp) did not influence transcription when placed 100-300 bp downstream of the normal mRNA initiation site for the *His3* gene, although it was able to function in either orientation with respect to the gene, when upstream of the mRNA initiation site (Struhl, 1984).

Clarification of the issue of UAS activity downstream of the site of the TATA element or transcriptional initiation site is pertinent not only to understanding of yeast gene regulation but also to the evolution of eukaryotic transcriptional machinery. A substantial body of knowledge indicates conservation of transcriptional machinery from lower eukaryotes to mammals; observations range from similarity in promoter organization to conservation of subunits of RNA pol and structural and functional relatedness of transcription factors (reviewed in Lin and Green, 1989). Yeast UASs are believed to be almost functionally identical to enhancers, which are the major determinants of the regulatory properties of a mammalian promoter. Enhancer elements are composed of one or more motifs, which can be repeated and are the binding sites for factors that act synergistically and can be cell specific (reviewed in Serfling *et al.*, 1985; Nomiyama *et al.*, 1987). Yeast UASs have been observed to stimulate transcription in mammalian cells and vice versa. Gene expression activation, in a Chinese hamster ovary cell line, has been observed when a promoter bore a yeast GAL4 binding site upstream of the transcriptional start site (Kakidani and Ptashne, 1988). Also, when yeast GAL4 was expressed in HeLa cells, transcription was initiated from several RNA pol II promoters that had GAL4 binding sites (Webster *et al.*, 1988). Similarly, vertebrate activators stimulate transcription in yeast, since transcription activation occurred when avian and viral *fos* oncogene products, expressed as fusion proteins in *S. cerevisiae*, were bound to DNA upstream of target genes (Lech *et al.*, 1988). In addition, the JUN oncoprotein, which causes sarcomas in chickens, activated

transcription in yeast with either its own or a heterologous DNA binding domain (Struhl, 1988).

In conclusion, this study provides strong circumstantial evidence for the presence of a yeast cell specific UAS, located within the *Bam*HI to *Sal*I fragment of *tet^r*. Further evidence of UAS identity would involve synthesis of synthetic oligonucleotides corresponding to the putative UAS, fusion of these to a reporter gene and monitoring of gene expression. In addition, mobility shift assays could be performed using yeast cell extracts. The perception that UAS sequences are inactive downstream of the TATA element of yeast promoters should be re-evaluated given: the deregulated transcription observed in a haploid yeast, when a *tet^r* fragment was placed downstream of the *GAL10* promoter, the limited number of studies which have addressed this capacity and most importantly, the high degree of conservation of transcriptional machinery of lower and higher eukaryotes.

Appendix 4. Description of anti-XLH5 antiserum

Since the full details of the anti-XLH5 antiserum used in this thesis are not published, the following appendix describes antiserum preparation and summarizes cross-reactivities of the antiserum with other vertebrate linker histone extracts. The anti-XLH5 antiserum used in this thesis was originally prepared for an investigation of the immunological relationship among vertebrate erythrocyte linker histone extracts of eleven species (Shay *et. al.*, 1988). Isolation of XLH5 cDNA expression clones was carried out using the antiserum (Rutledge *et. al.*, 1988).

Linker histones H1 and H5 were extracted from *X. laevis* erythrocytes with 5% PCA and subjected to exclusion chromatography in Bio-gel-P-150. Fractions enriched in XLH5 were then pooled, concentrated and injected, without yeast RNA, into guinea pigs. After the primary intradermal injection (20 μ g XLH5 per animal), several 10 μ g intradermal injections were administered at two and four weeks as well as two, three and four months. Antiserum was pooled from three animals five days after the last booster injection.

The immunodetection protocol used by Shay *et. al.* (1988) involved overnight incubation of a nitrocellulose filter, containing linker histone extracts, in a 1:100 dilution of the antiserum in PBS. Primary antibody was detected with protein A-conjugated horseradish peroxidase. Secondary antibody was detected by incubation of the filter in chloronaphthol. With this relatively high concentration of antiserum, cross-reaction with *X. laevis* H1A, B, C was observed, even though XLH5 is only 30-35% similar to these linker histones. Also, the antiserum showed intense cross-reaction with bull frog and leopard frog linker histones and moderate cross-reaction with the linker histones of other cold-blooded vertebrates (red-eared turtle, carp, axolotl, *Necturus*). However, cross-reaction with the satellite lysine-rich histone of trout was not observed.

This antiserum was shown not to cross-react with rat H1° or chicken H5. Since XLH5 is 62% homologous with chicken H5 and the central domains of these proteins as well as mammalian H1° are 90% homologous, it was concluded that the anti-XLH5 antiserum was deficient in antibodies against the conserved sequences in the central domains of H5/H1°. However, this same antiserum was also used to detect two partial XLH5 cDNA products, whose sequences include amino acids 11-108 and 8-196, with equal sensitivity (Rutledge *et. al.*, 1988; this thesis). These results suggest that the antiserum can detect at least 56% of the XLH5 coding region; a length that includes almost all of the central globular domain.

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