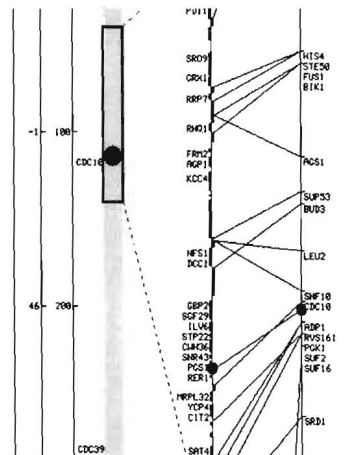


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GENOMIC DISTRIBUTION OF HISTONE H1 IN Budding Yeast (*Saccharomyces cerevisiae*)

Yeast Chromosome III



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Genomic Distribution of Histone H1 in Budding Yeast (*Saccharomyces cerevisiae*)

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Abstract

The linker histone H1 binds to the nucleosome and is essential for the organization of nucleosomes into the 30-nm filament of the chromatin. This compaction of DNA has a well-characterized effect on DNA function. In *Saccharomyces cerevisiae*, *HHO1* encodes a putative linker histone with very significant homology to histone H1. *In vitro* chromatin assembly experiments with recombinant Hho1p have shown that it is able to complex with the dinucleosomes in a similar manner to histone H1. It has also been reported that disruption of *HHO1* has little effect on RNA levels. A long-standing issue concerns the location of Hho1p in the chromatin and studies have shown using immunoprecipitation technique with anti-HA antibody, that Hho1p shows a preferential binding to rDNA sequences. In this project we have tried to confirm the above results in wild type cells, using immunopurified anti rHho1p antibody.

Introduction

The problem of how the fibers of chromatin are folded in eukaryotic nuclei has interested biologists and biochemists for decades. It has long been recognized that the histones play a major role in this folding (1). However, the distinctly different roles of the histones H2A, H2B, H3 and H4 on one hand, and the lysine- rich histones such as

H1 and its isotopes on the other, were not understood until after the discovery of nucleosomes (in early 1970's) which is known as the fundamental repeating unit of chromatin (2). Each nucleosome contains a core of histones around which DNA is wrapped and a fifth histone called the linker histone of which the most common is called histone H1 (2,3). Wrapping of 146bp of DNA about the histone octamer to form the core particle provides one level of folding (a compaction ratio of about 5:1), but this cannot account for the many thousand-fold condensation afforded by the DNA in the eukaryotic nucleus. The "string-of beads" structure observed in early electron microscopic studies (2,4,5) could also not satisfy the compaction requirement. It soon became evident that there must exist some level or levels of higher order folding of the chromatin fiber. Finch and Klug (6) showed that the extended nucleosomal filaments condense into irregular fibers of about 30nm diameter in the presence of even low concentrations (0.2mM) of Mg^{2+} . Later it was shown that increasing concentrations of either monovalent or divalent cations resulted in the progressive condensation of the fiber. The formation of well-defined fibers requires the presence of lysine-rich histones such as H1 (2,7,8). In eukaryotes transcriptional repression correlates in general with chromatin condensation. This can be either domain-wide or local, encompassing in the latter case just a few nucleosomes (10)

Research over the period of years has also suggested that not all eukaryotic cells have histones, for example dinoflagellates package their DNA with small basic proteins completely unlike histones (9). Certain eukaryotes are deficient in particular histones; for instance, yeast cells are apparently deficient in the type of histones that binds to DNA between nucleosomes (2).

A typical eukaryotic nucleus contains about 5pg of DNA, corresponding to about

5×10^9 bp. Associated non-covalently with the DNA are about 1pg each of 4 basic proteins called histones H2A, H2B, H3 and H4, always present in equimolar quantities. The histones are small proteins, each with a molecular mass about 104 kDa. A simple calculation shows that there are about two copies of each histone molecule for every 200bp of DNA. (11,23). In most cell types, there is also approximately one molecule per 200bp of a larger, very lysine-rich histone of the H1 class (14). Studies in the past decades suggests that digestion of chromatin DNA with micrococcal nuclease leads to the generation of a 'ladder' of DNA fragments on electrophoretic gels exhibiting a periodicity of about 200bp. Continued digestion produces chromatin particles containing about 146bp of DNA and octamer containing two copies each of four histones: $(H2A-H2B)_2 - [(H3)_2 (H4)_2]$. These particles are called 'nucleosomes', or more exactly 'nucleosomal core particles' and the 'core' histones (1,13). Whereas the lysine-rich histones were associated with the linker DNA between the core particles are called as 'linker histones' (1,13). The histones are distinguished from other proteins by several features. (i) Their highly basic character, (ii) a domain structure in which only the central part of the molecule adopts a structured confirmation while the N- and (in some cases) C-terminal tails are unstructured. (iii) Strong evolutionary conservation, particularly of H3 and H4 and (iv) the presence of non-allelic variants of each histone (except H4) in almost every organism. It is in the linker histones that the domain structure is most dramatically exhibited. Three distinct domains are discernible: a strongly basic, unstructured fragment at the N-terminal (nose) and a non-polar central globular domain (head) and a strongly basic unfolded domain at the C-terminus (tail) (15).

Discussion of the evolutionary conservation of the histones begins with the observation that the nose-head-tail structure of the members of the H1 family is so

strongly conserved that it is often used as a criterion for identification of the H1 proteins (13). But not all histones are highly conserved. The linker histones H1, H5 and H1° are highly variable proteins. In almost every species of eukaryote there exist non-allelic sequence variants in histones. The iso-histones differ in molecular mass, amino acid sequence and physico-chemical and immuno-chemical properties. All of the histones commonly exhibit such variants, with the exception of H4 (but even here two variants are known in *Tetrahymena*)(13). Though the chromatin of most cell types contains several different sequence variants of histone H1, the functional role of this heterogeneity are not well defined. (18). According to the studies done by Schulze *et al.* (15) using sub-type specific monoclonal antibodies, in the larval tissues of the midge, *Chironomus thummi*, there were variants of two types H1II-1, H1 II-2 and H1 III-1. They were reported to have similar amino acid sequences and appeared uniformly distributed in polytene interphase chromosomes (16). The total number of gene copies per genome for this type H1 histone is about 40 in *Chironomus th. thummi* and 50-60 in *Chironomus th. piger*. It had a divergent structure and was found in only a limited number of condensed chromosome sites.

Assembly of eukaryotic chromatin during the post replication period is a complex, multi step process. Apparently each pre-existing nucleosome dissociates into one H3/H4 tetramer and two H2A/H2B dimers, which recombine with newly synthesized histones and segregate randomly to either daughter DNA duplex (28). Additionally, new histone H3/H4 are deposited on the nascent DNA, while the majority of new H2A/H2B dimers are associated with non-replicated DNA, a new H1 is randomly distributed over chromatin (29). The higher order structure of newly replicated chromatin is probably unfolded. Maturation of this newly replicated chromatin is suggested to be mediated by histone H1, which is know to play an important role in

organization of the chromosome and of the higher order structure of chromatin. However, it is not yet clear when histone H1 is deposited during maturation. Earlier data obtained on fractionated chromatin is interpreted as indicating that nascent histone H1 appeared in chromatin only 10-20 minutes after DNA replication (19). Several approaches have been developed over the period of years, using MNase and immunoprecipitation of histone H1 cross-linked to DNA, Baryki *et al.* (35) developed a new immunochemical technique because of the uncertainties inherent in the above approaches, for analysis of protein content of nascent BrdUrd labeled chromatin. This technique includes reversible fixation of nuclei by formaldehyde followed by irreversible protein-DNA cross-linking by the DMS (dimethylsulphate) method, two-dimensional (2-D) electrophoretic fractionation, and immunodetection of the nascent BrdUrd-labeled DNA with anti-BrdUrd antiserum. The results suggest that histone H1 is deposited on newly replicated DNA simultaneously with or slightly after core histones. The experiments also demonstrate that while the histones are weakly bound to DNA at the early stages of chromatin replication, their globular regions seem to interact with DNA in nascent and mature chromatin with the same efficiency.

The nucleosome repeat length, the 146 bp of DNA associated with a histone octamer plus the linker DNA between adjacent nucleosome, can vary with species, cell type, physiological state and developmental stage, due to variability in the length the linker DNA (28). Nucleosomes are non-randomly placed with respect to DNA sequence. The role of linker histones in nucleosome positioning and alignment is not well understood. Early chromatin reconstitution experiments suggested that linker histones were responsible for spacing of adjacent nucleosomes and for spreading of nucleosome alignment along the chromosome (1). Studies conducted on H1/H5 depleted chicken erythrocyte chromatin showed that after an incubation for 16 hours

at 5°C in a buffer containing 0.7M NaCl, the regular ~212bp nucleosome spacing was lost. This was shown by the appearance, on digestion with micrococcal nuclease, of DNA fragments of nonspecific lengths and bands that are multiples of ~150bp, corresponding to closely packed nucleosomes. Such rearrangement or 'randomized' chromatin is largely a consequence of nucleosome 'sliding' (28).

Results show that H5 (and presumably H1) is actively involved in nucleosome spacing and that histone modifications are not necessarily required for the spacing mechanism to proceed.

Experiments were conducted in *Tetrahymena* macro-nuclear genome. Nucleosomes repeat length appeared to vary with the particular nucleosome. No changes in nucleosome position or repeat length were detected in the absence of histone H1 i.e. nucleosome positioning in a histone H1 knockout strain was indistinguishable from that in a strain with wild type histone H1.

Studies by Jeong *et al.* (31) reported histone H1 (or H5) dependent remodeling of chromatin structure that appeared to spread from a particular region of DNA to surrounding chromatin. Simple *in vitro* systems have provided some insight into the role of histone H1 in chromatin; for example, it was found that regular nucleosome spacing appeared to be required for the formation of higher-order chromatin structure by histone H1 (or avian erythrocyte H5). When histone H1 or H5 was re-associated with chromatin from which the linker histones had been gently removed and the internucleosomal spacing unperturbed, the chromatin seemed recondensed. In contrast when linker histones were added to chromatin containing irregularly spaced nucleosomes, non-specific aggregation and precipitation occurred at physiological ionic strength (30) Jeong *et al.* (31) examined extensive nucleosome alignment by histone H1 or H5 in some plasmid constructions, in a fully defined *in vitro* system.

They observed that plasmid pBR327 containing DNA insertions with lengths close to 300 bp permitted histone H5 to induce a remarkable degree of nucleosome alignment, and the same plasmid, not containing a DNA insert, permitted continuous alignment of only a few nucleosomes. These observations suggested that a necessary requirement in this system for histone H5 (or H1) induced nucleosome alignment on small (<4kb) circular plasmids may be that the total DNA length must be close to an integral multiple of the nucleosome repeat length generated.

A crucial question concerning structure(s) of chromatin fibers is the location of histone H1. The presence of this histone is, by virtue of its presumed location on nucleosomes out of the 30nm fibers (19).

According to neutron scattering experiments performed by Graziano. *et al.* (20) histone H1 is located in the interior of the chromatin 30nm fiber. Furthermore, chromatin 30nm filaments have ~6 nucleosomes per 11nm, with histone H1 at about the same radial location as the inner face of the nucleosome. This picture is in agreement with the solenoidal model as originally proposed by Finch, J.T and Klug, A. (21) and the fact that H1 is known to interact with nucleosome at the entry and exit points of nucleosomal DNA (20).

Several experimental approaches have been adopted over the past few decades to determine the exact location and function of H1. Alan *et al.* (22) showed the function of H1 as implicated in chromatin condensation (23) by preparing oligonucleosomes depleted by lysine-rich histone . The ability of peptides to compact the nucleofilament was also tested by observing the rate at which micrococcal nuclease digests the linker DNA between the core particles. (20,22). It is known that removal of H1 with consequent breakdown of the higher-order structure, leads to an 8-10 fold increase in

this rate (24) and H1 peptides have therefore been tested for their ability to restore the protection of linker DNA against digestion.

The location of H1 has been tested by nuclease digestion to the level of the core particle monomer. It has been proposed that the primary H1 binding site lies between 140 and 160bp (22) (the best current estimates are 146 and 165bp). There is a kinetic pause in nuclease digestion at 165bp and more of the H1 is lost only on subsequent trimming to 146bp (24).

Experiment conducted by James Allan *et al* (27) using peptides derived from calf thymus H1 and rat liver H1, comprising only the globular and COOH terminal domains of the intact molecule thus, lacking NH₂-terminal domains, was shown to be as effective as the complete H1 molecule in inducing high order chromatin structure. Thus, the X-ray crystal structure of the nucleosome core (46 and references there in) is the ordered nature of the histone amino-terminal 'tails' and the involvement of these 'tails' in nucleosome-nucleosome interactions. Although they are very long, the 'tails' show no secondary structure and they are visible for about 1/3 of their length. The amino terminal 'tails' of both H3 and H2B pass through minor groove channels of the DNA.

In yeast, biochemical efforts to definitively identify histone H1 were initially unsuccessful. However, as the sequence of the entire yeast genome became known (40), Landsman (39) asked whether an H1 homolog exists in yeast. Using the conserved globular domain of histone H1 as the basis for searching the *Saccharomyces* genome database, he identified an open reading frame (ORF) encoding a putative protein that has a very significant homology to the globular domains of many H1 proteins. The yeast H1, termed Hho1p, has an N-terminal

peptide that is lysine-rich followed by a well-conserved globular domain. No other linker histone molecules are known to contain two globular domains.

Ushinsky *et al* (41) and Patterson *et al* (38) performed comprehensive survey of potential phenotypes that might result from an *HHO1* deletion, but they too were unable to detect a phenotype particular *hho1Δ*. Patterson *et al* (38) tested the basal transcription levels in a minimal PHO5 promoter and showed no difference between wild type and *hho1Δ* cells. They also showed that Hho1p is not required for telomeric repression, nor is it required for efficient sporulation. However, they showed that purified recombinant Hho1p, like histone H1, was able to form a stable ternary complex with the reconstituted core dinucleosome *in vitro* at a molar ratio of one. They also reported that the reconstituted nucleosomes showed a kinetic pause at ~ 168 bp after micrococcal nuclease digestion of chromatin, as do nucleosomes associated with histone H1. *In vivo* bulk chromatin structure was unaffected by deletion of *HHO1*.

According to Puig *et al* (42) structural changes could be noted at a specific locus in chromatin following *HHO1* disruption. They chose a particular locus in the genome which includes *POT1* and *YIL161 w* that is located between two strongly micrococcal nuclease hypersensitive sites and is flanked by an array of 13 strictly positioned nucleosomes. They too were unable to detect a difference in the nucleosomes spacing between wild type and H1 knock out strains.

Despite all the above data that could indicate that Hho1p does not have a significant function in the cell, Spellman *et al.*(43), using gene array technology, reported that the only genes transcribed during S phase in yeast are *HHO1* and those genes encoding the core histones (*HTA1,HTA2* encoding H2A, *HTB1, HTB2* encoding H2B, *HHT1,HHT2*, encoding H3 and *HHF1,HHF2* encoding H4). As *HHO1*

transcription is coordinately regulated with the core histone genes, it is likely that Hho1p somehow functions in a coordinated fashion with the core histones.

Kanazin. *et. al.*(33) in an attempt to study the organization of histone H3 genes in soyabean, barley and wheat conducted experiments using specific probes for localizing individual gene copies and analyzing the genomic distribution of H3 variants across a range of genotype. Further, research conducted on the histone H1 genes of the dipteran insect, *Chironomus thummi*, reveal that H-1 variants H1 I-1 was found in a limited number of polytene chromosome bands, whereas other H1 histones H1II-1 and H1II-2 and H1III-1 were uniformly distributed in chromatin, indicating that there was an intimate functional difference between these two classes of H1 genes and their products. (34). On the same lines of research, studies by Chadwick and Willard (35) provided further information on the basis of several cloning and mapping experiments of an unusual variant of the core histone H2A called the MacroH2A, this investigation led to a more deeper insight, about the nucleosome composition of chromatin on the inactive X-chromosome and indicated that number of H2A variants were non-randomly distributed on the active and inactive X-chromosomes.

Investigations were also done on the variants of histone H1 and HMG proteins and their genes in dipteran insects; results have revealed different properties of DNA binding and intrachromosomal distribution. One of the variants of *Chironomus* was found only in minority of polytene chromosome bands and differed from the other H1 proteins of the same organism by genomic organization and by an inserted motif, the KAPKAP repeat that is also known to be present in single H1 variants of other, evolutionarily remote organisms. (36)

Thus, in *Saccharomyces cerevisiae*, *HHO1* encodes a putative linker histone with very significant homology to histone H1. The encoded protein is expressed in the nucleus, but has not been shown to affect global chromatin structure, thus a very useful method of studying this protein would be to study the interaction of this protein with the nucleic acids.

The presumption is that the living cell interactions of proteins and nucleic acids are preserved in the modified sample. A seemingly obvious approach for studying protein DNA interactions is Chromatin Immunoprecipitation (ChIP) (32).

Ducker and Simpson (37) have performed chromatin immunoprecipitation assays to study the structural role for Tup1p in repression and constrain models for organized chromatin in repressive domains, using Tup1p (a co-repressor that interacts with the N-terminal regions of H3 and H4) antibodies, which showed that Tup1p was associated with the entire genomic *STE6* coding region.

Significant results from this approach to chromatin organization *in vivo* at this time mainly involve the association of the Sir group of proteins with telomeric and silent mating type locus DNA (32).

Extension of this approach to other biochemically well-defined systems would lend credence to ChIP analysis of protein DNA interactions of functional as well as genomic significances.

Freidkin and Katcoff (45) in their study showed that in wild type cells, *HHO1* is both transcribed and translated and that the protein co-purifies with the core histones by replacing the chromosomal *HHO1* gene at its chromosomal location with the construct that produced a Hho1p-2HA fusion protein from the native *HHO1* promoter. They also performed a western blot on the whole cell extracts using an anti-HA antibody. They also measured its relative stoichiometry to the core histones in the cell,

finding that it is found in fewer copies in the cell than nucleosomes. Using DNA array technique they showed that disruption of *HHO1* does not have a transcription effect on a subset of genes. Using chromatin immunoprecipitation, with the DNA that had been immunoprecipitated with the anti-HA antibody, they directly probed where Hho1p might be located in the chromatin and showed that it is preferentially concentrated at the repeated sequences that encode rRNA.

In this project we further addressed the assignment of Hho1p as the linker histone in *Saccharomyces cerevisiae*. As previous studies have addressed to the query using an anti-HA antibody, which we thought would not be sufficient to localize Hho1p in chromatin, as we were not sure of the movement of the HA-tagged fusion protein we decided to use immunopurified rabbit anti Hho1p antiserum to obtain a qualitative estimate of the distribution of Hho1p in the chromatin.

Materials and Methods

***E.coli* strain and plasmid used for rHho1p purification and media**

pET20b (+)-*HHO1* in *E.coli* strain BL21 (DE3) containing the T7 lysozyme producing pLysS plasmid.

Yeast strains and media

Strain FY2 (MAT α , ura 3-52) was considered wild type genotypes and PEP1 (protease deficient strain) was used for total protein and nuclear protein extraction. Standard yeast YPD was used throughout.

Purification of Recombinant Hho1p (rHho1p)

Full length recombinant yeast Hho1p was expressed from the plasmid pET20b(+)-HHO1 in *E.coli* strain BL21(DE3) containing the T7 lysozyme producing pLysS plasmid. A 5ml volume of LB medium was inoculated with an overnight colony of the transformed *E.coli* strain, and grown to OD₆₀₀ of 0.7. The cells were pelleted (2000×g, 5min, 4°C), and inoculated into 1 L volume of LB. The culture was incubated at 37°C with continuous agitation until OD₆₀₀ of 0.7 was reached. Thereafter, Hho1p was induced with 0.4mM isopropyle-1-thio-13-D-galactopyranoside (IPTG) and the incubation continued for the times indicated in the text. The cells were collected by centrifugation (2000×g, 5min, 4°C). The supernatant was loaded onto a nickel-agarose column

(Novagen His Bind Kit) of 1 ml bed- volume, pre-equilibrated in binding buffer. The column was washed with 25ml binding buffer, followed by 15ml of wash buffer (20mM imidazole, 500mM NaCl and 20mM Tris-Cl pH 7.9). The protein was eluted in 1M imidazole, 200mM NaCl, 20mM Tris-Cl pH 7.9 and 1 ml fractions were collected. Protein containing fractions were identified by SDS-PAGE electrophoresis. Fractions containing the relevant protein were pooled and concentrated into 10mM sodium phosphate (pH 7.0), 0mM NaCl, 0.5mM phenylmethylsulfonyl fluoride (PMSF), using a Centrex 30000 Da Column. Which would retain all the proteins of the molecular weight above 30000Da. The concentrated sample after centrex was processed on a CM-Sephadex cation exchange column (1 ml bed volume) pre-equilibrated with the same buffer. The column was then developed with 10 ml of a 0mM to 1000mM linear gradient of NaCl in 10mM sodium phosphate (pH 7.0), 0.5mM PMSF. 1 ml fractions were collected. The eluted protein fractions were analyzed on 12% SDS-PAGE gel and coomassie stained as per standard procedures.

Determination of Antibody Titer by Enzyme linked immunosorbent assay (ELISA)

The antigen was diluted in PBS and coated the polysorb ELISA plate with 100 μ l Antigen (10 μ g/ml) covered it with cling wrap and incubated overnight at 4°C. The next day, tipped off the excess antigen and washed the wells 3X with TBS TWEEN (100mM Tris-HCL pH 7.5, 137mM NaCl, 0.1%(v/v) Tween 20). The wells were then blocked using 200 μ l 3% BSA TBS TWEEN (100mM Tris-HCL pH 7.5, 137mM NaCl, 0.1%(v/v) Tween 20 and 3%(w/v) BSA (Bovine serum albumin) and incubated for 1 hour at room temperature. The plates were then reverted so as to tip-off excess of block solution and washed 3X with TBS TWEEN. Then serial dilution of primary antibody (anti rHho1p) was performed from 10⁻² to 10⁻⁵ and 100 μ l of each was added to the rows of wells. The first row was used as blank without the primary antibody and incubated at room temperature for 1 hour. Excess antibody was tipped out and washed the wells as before 3X with TBS TWEEN and to the plates a 1:3000 dilution of secondary goat anti-rabbit alkaline phosphatase conjugate was added to each of the wells (all the dilutions were performed in TBS TWEEN) and incubated for an hour at room temperature. Again excess of secondary antibody was tipped out and washed 3X with TBS TWEEN and performed another wash in 10% Diethanolamine, 0.5mM MgCl₂ to equilibrate and added 100 μ l of substrate (10mg pNPP in 10mls 10%diethanolamine/0.5mM MgCl₂). First stable readings of a positive reaction, which is yellow in color, were read after half an hour using the 405nm filter of the Titretek Multi-Scan Machine. Another set of readings were tread after an over night incubation.

Protein Extraction and Western Blotting

Total yeast protein was extracted as described in (Horvath and Riezman, Yeast, 1994;Gottschling Lab). Briefly, yeast cells were grown overnight ($\sim 1 \times 10^7$ cells/ml; $A_{600} = 0.7$) and collected the 1.5 ml cells (adjusted the volumes according to the cell density of cultures) in 1.5 ml microfuge tubes and pelleted the cells for 1 min. at $14000 \times g$. The cells were then washed with water and collected again by centrifugation and resuspended in 100 μ l sample buffer (0.06 M Tris-HCl, pH 6.8, 10%(v/v) glycerol, 2%(w/v) SDS, 5%(v/v) 2-mercaptoethanol, 0.0025%(w/v) bromophenol blue). The prepared samples were heated at 95°C for 5 minutes and centrifuged at $14000 \times g$ for 5 min. and loaded 20 μ l per lane on 12% SDS polyacrylamide gel and coomassie stained as per standard procedures.

Yeast nuclei protein was extracted primarily using Yeast Nuclei Protocol (B.Strahl, personal communication). A volume of 1 L of yeast cells were grown to mid-log phase and pelleted at 5000rpm for 5min. and the cells were then washed briefly with ice-cold water and resuspended in 50 ml spheroplasting buffer (1M sorbitol, 50mM potassium phosphate pH 6.5, 14mM β Mercaptoethanol) and pelleted again at 5000g for 5 min. The pelleted cells were again resuspended in 10 ml spheroplasting buffer with 1 ml (at 10 mg/ml) zymolase. And incubated for ~ 1 hour until the cells are spheroplasted and the spheroplasted cells were again pelleted at 5000 rpm, 5 min at 4°C . The pelleted cells were washed in 10 ml spheroplasting buffer and resuspended in 10 ml lysis buffer (18% Ficoll 400, 20mM potassium phosphate buffer pH6.8, 1mM MgCl_2 0.5 mM EDTA, 1 mM PMSF, 1 μ g/ml leupeptin/pepstatin.). The cells were then subjected to 20 strokes with dounce homogeniser (pestleB) and chilled on ice for 10 mins. and again pelleted the debris for at 5000 rpm for 10 mins. The supernatant was collected and transferred to a fresh tube and pelleted the nuclei at 18000 rpm for 30 min. The nuclei were resuspended in 10 ml NP buffer (0.34M sucrose, 20mM Tris-

HCl pH7.4, 50mM KCl, 5mM MgCl₂, 1mM PMSF, 1µg/ml leupeptin/pepstatin. Then again the nuclei were pelleted in 2ml cushion buffer (1.7M sucrose in NP buffer) at 15000rpm for 30 mins and resuspended the nuclei finally in 1ml NP buffer for storage at -20°C.

The proteins (rHHO1p, total protein, and nuclei protein) were first analyzed on a 12% SDS-PAGE gel at 150V for 2 hours and later soaked the gel, ECL nitrocellulose membrane, and whatman papers, in protein transfer buffer for about 20 min.

The electro blotting transfer 'system' was casted and the transfer was performed overnight at 4°C at 30 volts.

After the overnight transfer the membrane was blocked in the blocking buffer (0,1 ml TWEEN 20, 2.5 g fat free milk powder, 50 ml PBS) overnight at 4°C and later on washed the membrane with TBS-T (20mM Tris-Cl pH7.6, 137mM NaCl) thrice (one 15 min. wash and 2 ten min. washes with a change of buffer every time.). The membrane was then incubated in 10ml (1:1000) dilution the primary antibody (anti rHho1p antibody) (all dilutions were performed in TBS-T) incubated the blot for 1 hr. at room temperature. and again washed the nitrocellulose 3 times with TBS-T. A 1:1000 dilution of the secondary antibody (Peroxidase labeled anti-rabbit IgG) was performed in the meanwhile and after the washes the blot was incubated with gentle shaking for 1 h at room temperature. The blot was then finally washed 3 X with TBS-T as before and proteins were visualized with the ECL Western Blotting Kit. (Amersham) by mixing the same amount of reagent 1 and 2, of the kit by pipetting the mixture over the blot and letting it affect for 1 min. extra mixture was removed from the blot and it was wrapped in cling film and exposed the first film for 15 sec to view the signal under safe light. The blot was then developed using normal procedure for developing autorads.

Immunopurification of Anti-Rabbit-Hho1-IgG

The resin was prepared by weighing out required amount of sepharose (CNBR activated Sepharose 4 B Amersham Pharmacia) and washed the sepharose with several aliquots of 1 mM HCl for 15 min. (70 ml for 1ml sepharose). Meanwhile the protein to be coupled was dissolved in 0.1 M NaHCO₃ pH 8.3 0.5 M NaCl. About 5-10 mg protein/ml gel was used, mixed gently overnight at 4°C and washed away the excess ligand with 5 gel volumes of coupling buffer(0.1MNaHCO₃,0.5M NaCl pH 8.3) (Blocked remaining active groups with 0.1 M ethanolamine pH 8.0 and let it stand for 2 h. and washed the beads 3X with alternating pH: wash buffer (0.1M acetate buffer pH 4 containing 0.5M NaCl) and (0.1M Tris-Cl pH8 containing 0.5 M NaCl). The beads were then washed with 10 ml PBS and added the serum (about 1ml) and 4 volumes of PBS (80mM disodium hydrogen orthophosphate abhydrous, 20mM sodium dihydrogen orthophosphate, 100mM sodium chloride, pH7.5) to the beads and rolled overnight at 4°C. and next the beads with 10 ml PBS.

The resin was into a column carefully eliminating air-bubbles and the antibody was eluted with 0.1 M Glycine pH 2.4 in 1 ml fractions.

Chromatin Immunoprecipitation

Immunoprecipitation of DNA was carried out essentially as described in (44). Aliquots from overnight cultures of FY2 were inoculated into 110 ml of yeast (FY2) were grown in YPD medium to an OD₆₀₀ of ~ 1.0 to 1.5. Formaldehyde (Sigma) was added to the culture medium to a final concentration of 1% and mixed slowly for 15 min on a MAXI rotator at room temperature. The reaction was then quenched with glycine to a final concentration of 125 mM. the formaldehyde-fixed cells were

harvested at 3000 g at 4°C for 5 min. the fixed cells were washed two times with 10 ml each cold PBS. And resuspended in 2 ml PBS and divided between two microfuge tubes. Which were centrifuged again at 3000 g at 4°C for 5 min. to harvest the cells. Later the harvested cells were resuspended in 400µl lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1mM EDTA, 1 % Triton X-100, 0.1% sodium deoxycholate and a cocktail of protease inhibitors as described in (44)).

To the above mixture 0.4 g of glass beads (0.5 mm diameter acid washed) was added and vigorously vortexed at high speed for 50 min. at 4°C. The mixture was then spun briefly to concentrate the lysate at the bottom of the tube the cell debris formed the pellet and an additional 250µl of lysis buffer was added and again vortexed and both the supernatants were combined.

The lysate was sonicated (using Virsonic Digital Sonicator manufactured by United Scientific, using the micro tip at power output of 4) on ice using a fine probe with short 5 sec pulses with a rest of 10 sec to a total of ~20 sec (45), taking care that the lysate did not become warm. The lysate was spun briefly at 17000 g for 15 min at 4°C to clarify the sonicated material. And saved 200 µl of lysate for preparing total genomic DNA. The supernatant (~1.1 ml) was transferred to a pre-chilled tube (~ 14 ml) and 9 volumes (9.9 ml) of ice- cold IP dilution buffer (0.01%(w/v), 0.1%Triton X-100 (v/v), 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) supplemented with 0.5 mM PMSF, 0.8µg/ml pepstatin and 0.6µg/ml leupeptin was added and incubated for 15 min.

The diluted the lysate was clarified by centrifuging at 8000 g for 10 min at 4°C. and decanted the supernatant to another tube and stored on ice and saved 300µl of solubilized chromatin solution (Input/WCE) and the remainder were aliquoted into microfuge tubes and anti-Hho1 antibody (25µg/ml) was added and slowly allowed to

mix overnight at 4°C. to the tubes 2µg sonicated λ genomic DNA was added to block non-specific binding of DNA and then added 80 µl Protein-A-Sepharose-CL-4B beads (Amersham Pharmacia) and incubated for 3 hours with gentle mixing at 4°C. the beads were spun briefly for 1 min. at 17000 g at 4°C to concentrate them and here an aliquot of 300 µl supernatant (unbound fraction) was retained and aspirated remaining supernatant carefully the beads were carefully washed 3X in 10 mM Tris-Cl pH 8, 0.25 M LiCl, 0.5% NP-40, 0.5% Na deoxycholate, 1mM EDTA spinning for 1 minute at 17000 g at 4°C and finally washed again with 10 mM Tris-Cl, pH 8, 1mM EDTA. and added 500µl elution buffer (1% ultra pure SDS, 0.1M NaHCO₃) to the beads and vortexed briefly and incubated for 15 minutes at 4°C and centrifuged for 1 minute at 17000 g at 4°C the supernatant was transferred(avoiding the beads) and repeated again the same with the pellet and combined both the supernatants. To which 40µl (1/25 volume) 5M NaCl to elute IP's and (1/100 volume) was added. And 3 µl 5M NaCl was added to solubilized chromatin solution and unbound fractions and added 200 µl of 50 mM Tris – HCl pH 8, 10 mM EDTA, 1% SDS and heated at 65°C over night to reverse the cross-links. Following day the tubes were briefly spun to remove evaporated liquid from the lid and to each one of them added 2 volumes absolute ethanol and precipitated the DNA at –20°C overnight.

The next day the samples were centrifuged at 17000 g for 20 minutes at 4°C. Carefully the supernatant was aspirated and washed the pellet with 70% (v/v) ethanol, vortexed and centrifuged. Then the pellets were dried briefly and resuspended in 100µl TE (10mM Tris-HCl, pH8.0 and 0.1 mM EDTA) and allowed to rehydrate at 4°C.

At this point added DNase-free RNase A (20 μ g) was added and incubated the samples at 37°C and 25 μ l 5X proteinase K buffer (50mM Tris, pH8, 25mM EDTA, 1,25% SDS) was added to each sample and mixed well and then added 1.5 μ l proteinase K solution. The samples were then incubated at 42°C for 1 to 2 hours. Later 175 μ l TE was added to the immunoprecipitated material (300 μ l final volume) and 275 μ l TE to the total samples and extracted the immunoprecipitated material once with an equal volume of 300 μ l freshly prepared PCI (phenol: chloroform: isoamylalcohol; 25:24:1) and then with an equal volume of chloroform. And extracted input and unbound fractions twice with the equal volume 400 μ l of PCI and then twice with an equal volume of chloroform back extracted the organic phases with 100 μ l TE and combine with the primary aqueous phase. Added 5 μ g glycogen to all samples, followed by 1/10 volume of 3M sodium acetate and 2 volumes absolute ethanol and precipitated the DNA overnight at -20°C and centrifuged the samples at 17000g for 20 min at 4°C the pellet was washed with 70% ethanol, vortexed and recentrifuged and air-dried briefly and resuspended all samples in TE and allowed DNA to rehydrate at 4°C prior to analysis. PCR amplification was carried out in 50 μ l volume with 0.5 μ M final concentration of each primer (45) (forward primer nt201-224 of 25S rDNA transcript: 3'AGGACGTCATAGAGGGTGAGAATC 5') and (reverse primer nucleotide 585-608 of 25S rDNA transcript: 3' TTGACTTAACGTCGCAGTCCTCAGT 5'), 150 μ M final concentration of dNTP's, 5 units Taq polymerase, 1X Taq buffer, and 1ng DNA template and appropriate amounts water were mixed together. Standard cycling program was used with 2 min initial denaturation at 95°C followed by 25 cycles with 30 sec at 95°C denaturation, 30 sec at 55°C annealing and 60sec at 72°C elongation and final extension step of 5 min at 72°C. The product size was expected to be 408

bp. After the completion of amplification, 15µl of each was loaded along with the size standard on 1% agarose gel in 1XTBE buffer 0.1µg EthBr/ml for 2hours at 90 V at room temperature. The results were observed under UV

RESULTS

Purification of Hho1p and its antigenic nature

As Hho1p has significant sequence homology to histone H1 in other organisms, it has been completely established that it is yeast histone H1. Transcription during the S phase of the cell cycle is unique to core histones and *HHO1*, strongly supporting this contention.

Histone H1 is thought to be associated with all or most nucleosomes in higher organisms, but studies conducted by Freidkin and Katcoff (45) reasoned that this might not be true for Hho1p since cells lacking Hho1p did not show a difference in nucleosome spacing, thus concluding that Hho1p is unlikely to be directly responsible for general chromatin packing in yeast. DNA array experiments comparing expression of all ORFs in wild type and *hho1Δ* strains showed that limited number of transcripts are affected by the absence of Hho1p in the cell, which may explain the lack of an observed phenotype in *hho1Δ* cells. This information was used by researchers to try and locate the relatively low numbers of Hho1p molecules in the chromatin using chromatin immunoprecipitation technique, with an anti-HA antibody the data revealed a preferential binding of Hho1p to rDNA.

Thus in this project we have tried to re-establish the location of Hho1p molecules in chromatin using anti-rHho1p antibody, as we reasoned that, the mobility and specificity of an anti-HA antibody might not be accurate in terms of its immunoprecipitating properties.

We therefore isolated recombinant Hho1p (rHho1p) by over expressing *E.coli* strain BL21 (DE3) The isolated Hho1p was further purified by metal chelation chromatography as shown in Figure-1. The actual molecular weight of Hho1p was determined in previous reports to be 30 k Da and the recombinant protein was seen to run on a SDS-PAGE gel just near to 31 k Da. Further the relevant protein fractions were processed on a CM-Sephadex cation exchange column to get rid of the contaminant proteins, as seen in Figure-2. The purified protein (1 mg/ml) was used for immunization of the rabbit and the pre-immune and post-immune serum were tested for determination of Antibody titre using ELISA (Enzyme linked immunosorbent Assay). The antibody titre was determined to be 1 in 48000 immune serum as shown in Figure-3(a) in the post immune serum and as seen in figure 3(b) negligible specificity to antigen rHho1p was observed in the pre immune serum. Thus, confirming that rHho1p had a very high antigenic capacity in Rabbit.

Characterization of rabbit anti-rHho1p antibody

Further as we found a very high antibody titre, we asked a question towards its specificity to the antigen rHho1p in a purified state, as well as in a whole cell extracts of total yeast protein. To study the following total protein was extracted from a yeast protease deficient strain which were analyzed by running on 12% SDS-PAGE gel, and further coomassie stained using standard procedures as seen in Figure-4 (A) further the gel was western blotted and probed using the anti-rHho1p antibody and the signals were studied using the chemiluminescence detection system., using anti rabbit

IgG with a horseradish-peroxidase conjugate as secondary antibody. Results indicated that anti-rHho1p antibody did recognize the yeast histone-H1 in the total yeast protein extracts with some cross binding and reactivity to other proteins as well, so we decided to immunopurify the antibody to minimize the cross-reactivity.

Immunopurification and Characterization of anti-rYH1 antibody

Research has indicated that the outcome of the chromatin immunoprecipitation assay depends critically on the quality of the antibody used and that they should possess a high-affinity for the antigen and should cross-react minimally with other proteins. Thus to minimize the risk of potential problems due to non-specific reactivity of polyclonal antiserum, the antibodies were immunopurified/affinity purified. This was achieved by absorption of the antigen rHho1p used for immunization to a CNBr activated sepharose column to immunopurify and elute the antibodies which were analyzed on a 10% SDS-PAGE gel, to see whether there were any antibodies and as seen in Figure-5 the band revealing the antibodies is below the 200,000 Da myosin band, indicating the reported molecular weight of and IgG molecule to be ~150,000 Da To further characterize its purity we performed a western blot with immunopurified anti rabbit rHho1p against total yeast protein extracts and purified rHho1p and as seen in Figure-4(B), after the immunopurification it was observed that the cross-reactivity was minimized.

Chromatin Immunoprecipitation of DNA bound to HHO1 in wild type FY2 strain

Investigators have previously shown that recombinant glutathione S-transferase(GST)- Hho1p-2HAp could be immunoprecipitated with commercially prepared anti-HA antibody .It was also reported that the DNA bound to Hho1p-2HAP does not show preferential binding of Hho1p to differentially expressed genes and that it showed preferential binding to rDNA sequences, indicating the localization of Hho1p in chromatin.

Using this information we asked a question whether Hho1p could be immunoprecipitated in the wild type yeast cells with rabbit anti rHho1p antibody immunopurified by us. And further we also tried to investigate the localization of Hho1p in chromatin, whether it would show a preferential binding to rDNA sequences as reported earlier as well as other specific locations in the chromatin.

Thus we performed a ChIP assay with immunopurified rabbit anti rHho1p antibody and purified and eluted the Immunoprecipitates using protein-A-sepharose beads. To analyze the presence of immunoprecipitates (IP), we performed a western analysis of the Immunoprecipitated sample, the solubilized chromatin sample(input or total samples) and the unbound fractions. The goal of this experiment was primarily to characterize the immunoprecipitated DNA, thus it was necessary to control the efficacy of the precipitation by analyzing the protein content of the input, the IP and the unbound fractions. For this purpose protein concentration in each of the samples was determined by Bradford assay and analyzed on 12% SDS-PAGE which was western blotted as seen in Figure-6, the results showed no significant difference in the intensities so as to conclude that the amount of protein present in the immunoprecipitated samples was more compared either to the input or whole cell extract and the unbound fraction, raising several questions in terms of the nature of extent of immunoprecipitation of Hho1p and also the cross linking of the protein to the

DNA and its enrichment, but, it did conclude that Hho1p could be immunoprecipitated with anti rabbit Hho1p antibodies.

PCR Analysis

As we concluded that we had DNA fragments with desired protein Hho1p, we further investigated to test the enrichment of the immunoprecipitates compared to other portions of the genome, and thus we used PCR in which the immunoprecipitated material was used as template for amplification primarily with 26rDNA primers as Hho1p is reported to have shown a preferential binding to this region as seen in Figure-7 till today our results did not show any enrichment or preferential binding of Hho1p to rDNA sequences but a more indepth study and investigation is being done to make any conclusions.

Discussion

Previous work has been unable to establish a phenotype for *hho1Δ* cells. Though it was shown that *HHO1* is transcribed (43) the existence of Hho1p in the cell when transcribed could not be established. Freidkin and Katcoff (45) reported that they could detect Hho1p in cellular extracts with an anti HA antibody. They also observed that after Coomassie staining the band had a higher mobility when it was tagged with two copies of HA. To address this issue we asked whether we could detect Hho1p in cell extracts using anti rHho1p antibody in wild type (WT) extracts and use the data to localize Hho1p in chromatin using an immunoprecipitation technique.

We performed experiments using cell extracts from wild type yeast cells and over expressing *E.coli* strain BL21 (DE3) to purify rHho1p and we were able to identify a

single band at the appropriate molecular weight by performing western blots with anti rHho1p antibody, supporting its identification in the WT cells.

We noted that the anti Hho1p antibody showed some cross reactivity to other proteins as well, so to minimize the cross-reactivity we immunopurified the antibody using affinity purification and we observed that the cross reactivity was significantly minimized. We conclude that it is necessary to immunopurify the antibody to allow application in ChIP assays.

Previous analyses of genome-wide expression profiles in a synchronous mid-log cultures of (43) WT and *hho1Δ* strains showed that the levels of a limited number of transcripts were affected by the absence of Hho1p in the cell. This result may be the lack of a readily observable phenotype. Further research to locate the relatively low numbers of Hho1p molecules in the chromatin using the chromatin immunoprecipitation, have shown that Hho1p molecules were preferentially found at the repeated genes encoding rRNA(45)

However this study made use of a anti-HA antibody and a Hho1p-HA fusion protein. There is some question as to whether this fusion protein positions identically to the native H1 molecule. To overcome this uncertainty we decided to make use of antiserum directed against unmodified Hho1p to investigate functional distribution of this protein in the nucleus. We also performed a Western analysis of the immunoprecipitated samples along with the input and unbound fractions to estimate the protein composition of the immunoprecipitated fractions and found that Hho1p was present in all the three samples (input, unbound and IPed) This result may indicate that only a fraction of the Hho1p is bound and precipitated by the anti-serum due to structural unavailability of the H1 epitope or due to excess H1 in the reaction tubes as compared to the amount of the antibody molecules available. To confirm this we

calculated that in a reaction tube of 1.5 ml $\sim 975 \times 10^9$ nucleosomes were present to which 5.85×10^{-11} moles of Hho1p is associated and the amount of antibody added to the reaction mixture to immunoprecipitate was 2.5×10^{-12} moles/15 μ l of antibody used at a final concentration of 25 μ g/ml, which was not sufficient to pull all the Hho1p molecules, as result large quantities of Hho1p molecules might have been left unbound. Alternatively, if the cross-linking efficiency is low, Hho1p may redistribute between antibody bound pellet and the supernatant, resulting in the equal distribution of signal that was observed. We therefore did not expect a high enrichment of the Hho1p bound chromatin, but we performed the PCR analyses (which is currently underway) using primers specific for 25SrDNA regions previously shown to be associated with Hho1p-HA(45). We have designed and purified primer sets for the amplification of the other possible locations like the centromeric regions, the telomeric regions, recombinant enhancer regions and MAT α regions and inducible and constitutively expressed genes such as GAL1 and ACT1. The results from this investigation should give an insight into the functional distribution of linker histone H1 among structurally different regions of the genome and contribute to the understanding of the function of this histone in the eukaryotic nucleus.

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Purification of rHho1p: Metal chelation chromatography

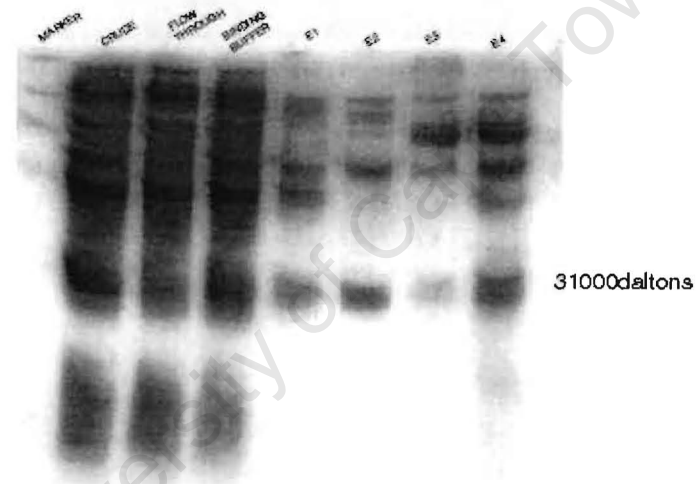


FIGURE 1: PURIFICATION OF YH1 AFTER PASSING THROUGH Ni-AGAROSE COLUMN:
Lane 1, marker (mol. wt. 200, 116, 25, 97, 4, 66, 2, 45, 31, 21, 5, 14, 4, 6, 5 kDa, respectively);
Lane 2, crude extract, lane 3, Flowthrough, lane 4, fraction from binding buffer, lane 5-8
rHh1 elution fractions.

Purification of rHho1p: CM-Sephadex Column

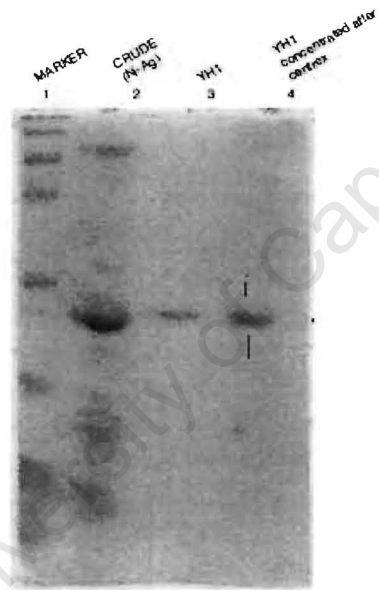


FIGURE:2 PROTEIN PURIFICATION AFTER CM-SEPHADEX. Lane,1 MARKER, Lane,2 Crude after Ni-Ag Lane,3. Purified rHho1p. Lane. 4 Concentrated rHho1p after Centerx.

ELISA: Pre Immune serum

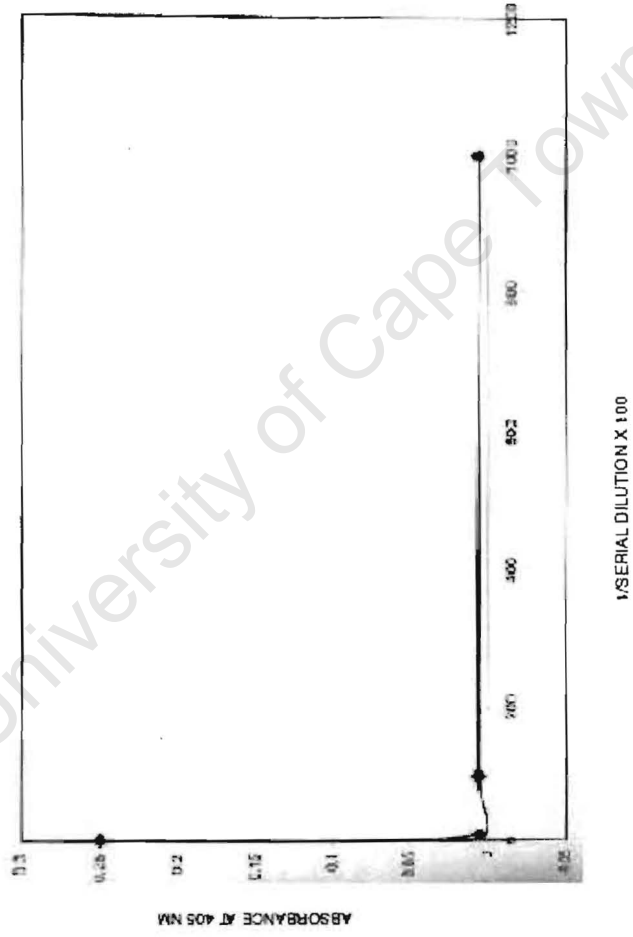


FIGURE 3(a) : PROFILE OF ANTIBODY TITRE IN PREIMMUNE SERUM

ELISA: Post Immune Serum

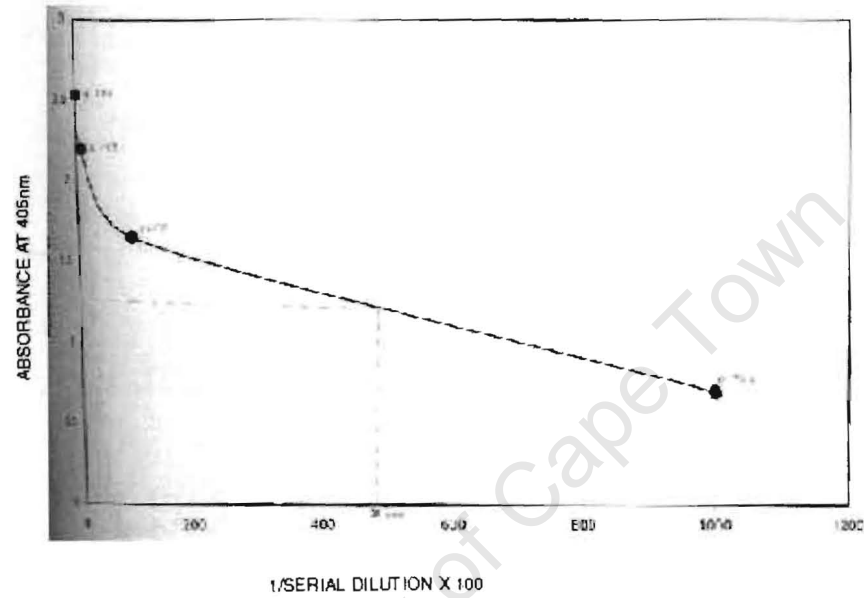


FIGURE 3(b): PROFILE OF ANTIBODY TITRE IN POST IMMUNE SERUM
(an average of the readings was taken)

Immuno purification of anti-Hho1p antibody

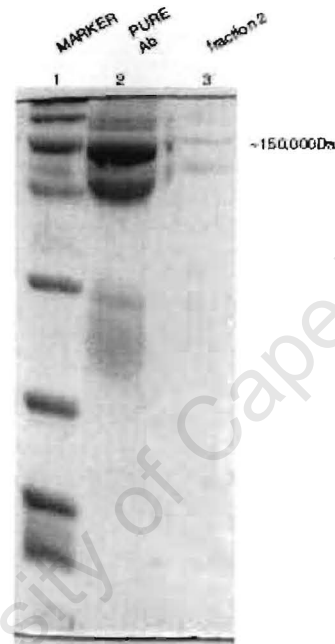


FIGURE 5: 10% SDS-PAGE GEL OF IMMUNOPURIFIED rHho1p ANTIBODY
Lane 1: Standard Molecular weight marker, Lane 2: immunopurified antibody from CNBr activated sepharose column, show a molecular weight of ~150kD.

Western Analysis: rHho1p in Whole cell extract from WT cells.

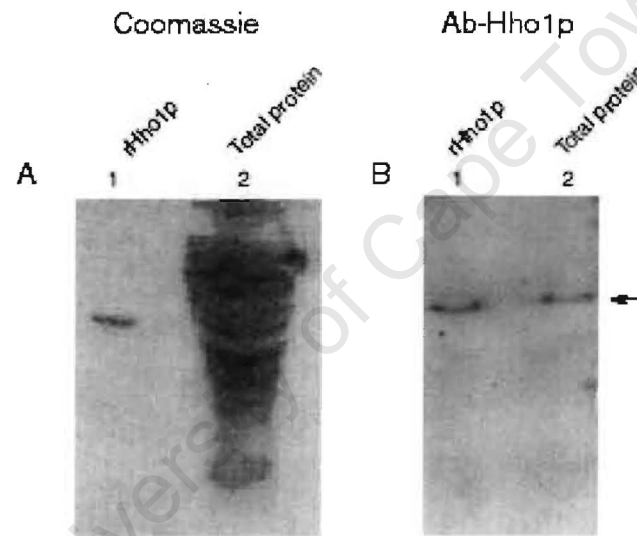


FIGURE 4:(A) 12%SDS-PAGE gel, Lane1:purified rHHO1p, Lane 2:Total yeast protein extract from wild type cells. (B) Western Blot,Lane 1: purified rHHO1p, Lane 2:Total yeast protein extract from wild type cells.

Western Blot After Chromatin Immunoprecipitation

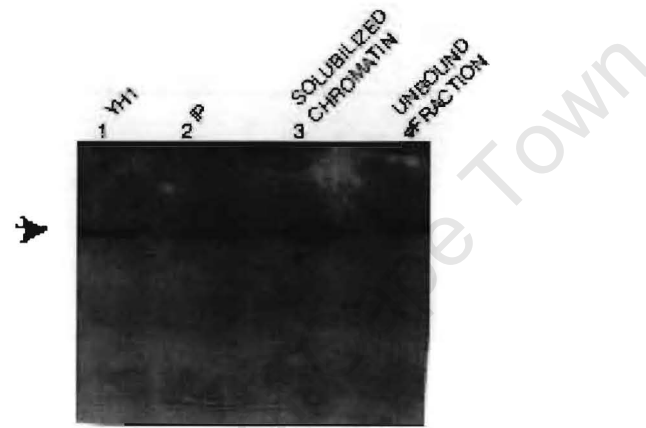


FIGURE:6: WESTERN BLOT AFTER Chromatin Immunoprecipitation: Lane 1 purified rH1p
Lane 2 Immunoprecipitated DNA bound to HHO1p, Lane,3 Solubilized Chromatin Solution,
Lane,4, Unbound fraction

PCR ANALYSIS

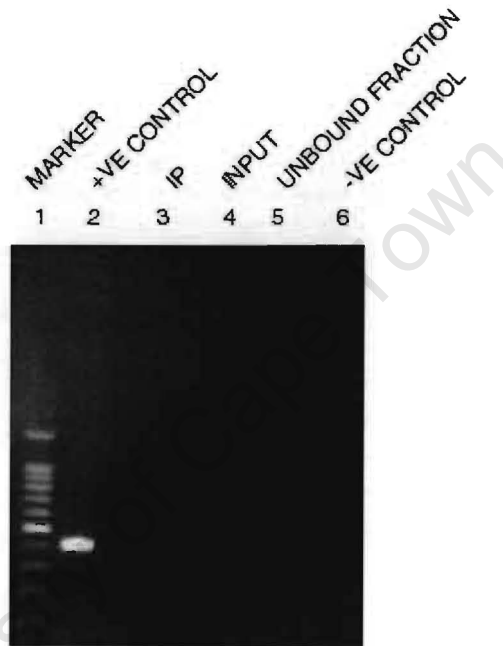


FIGURE 7: PCR ANALYSIS: LANE 1 Mol.Wt. Marker(100bp), Lane 2 +ve Control, Lane 3 Immunoprecipitated fractions., Lane 4 Input fraction, Lane 5 Unbound fraction, Lane 6 -ve Control