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Abstract

Genes are regulated in different ways e.g. via transcription factors, regulatory sequences and epigenetic mechanisms. One of the epigenetic mechanisms is the change in localisation of genes in the cell nucleus during regulation. In this study the *lacO*/LacR-GFP recognition system was successfully used to visualise clustered nitrogen starvation genes in *Schizosaccharomyces pombe*. As a reference point Pom152-YFP was crossed into the strains. The upregulation of selected genes during nitrogen starvation was verified by RT-PCR. By this, conditions to study the localisation of genes induced by nitrogen starvation in *Schizosaccharomyces pombe* were created.

Keywords

Nitrogen starvation genes, clustered genes, gene localisation, *Schizosaccharomyces pombe*, *lacO*/LacR-GFP recognition system

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Creating conditions for studying localisation of clustered genes induced by nitrogen starvation in *Schizosaccharomyces pombe*

Sara Lyckman

Sammanfattning

I en frisk organisms celler finns alla gener, men alla är inte aktiva samtidigt. Med dagens intensiva forskning ökar kunskapen om genernas funktion, men även förståelsen för hur generna regleras. En reglerande faktor verkar vara genernas position i cellkärnan – olika lokalisering av en och samma gen kan påverka dess aktivitet.

Detta arbete syftade till att undersöka om gener i jästsvampen *Schizosaccharomyces pombe* som aktiveras av kvävesvält flyttar på sig i cellkärnan då de aktiveras. För att kunna se lokaliseringen av generna i mikroskop så märktes DNA nära generna in med fluorescerande färg och för att få en referenspunkt så märktes cellkärnans hölje in med en annan färg. Tanken var sedan att fotografera celler och mäta avståndet mellan genernas position och cellkärnans hölje i vanligt växande celler och jämföra dessa värden med avstånd uppmätta i celler som växt i kvävefattigt medium och se om det fanns någon signifikant skillnad.

Några av genernas aktivitet undersöktes under kvävesvält och en uppgång i aktivitet kunde bekräftas. Inmärkningen av generna lyckades bra men på grund av tidsbrist så utfördes inte några mätningar i mikroskopet. Arbetet resulterade dock i goda förutsättningar för fortsatta studier av lokaliseringen av gener som aktiveras under kvävesvält.

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1 Introduction

The "cracking" of the genetic code opened a door into a new big room in the house of science. The first genome to be published was *Haemophilus influenzae* in 1995. To date, 409 genomes have been published including the human genome, and there are currently 1 718 ongoing genome projects [1]. We have not yet turned on the light completely in the "gene room" concerning e.g. the function, evolution, regulation and interaction between genes but we are on our way. New techniques have made it possible to investigate more and more of the unknown facts and there are a lot of studies concerning the functions of genes. When the functions are known the next question is how genes are regulated and this field is subject to a lot of research too. Genes are regulated on several different levels e.g. via transcription factors, regulatory sequences and epigenetic mechanisms such as modifications of histones and DNA, different chromatin states and nuclear architecture [2].There is an extensive cooperation between these levels.

This study will focus on the epigenetic mechanisms and particularly on exploring if the localisation of genes in the cell nucleus affects their level of expression. But first, a brief background about chromatin and nuclear architecture.

1.1 Euchromatin and heterochromatin

The eukaryotic genome is highly organised. The DNA helix is wrapped twice around a histone octamere complex consisting of subunits H2A, H2B, H3 and H4. Histones are proteins that consist of a globular part and a more flexible tail at the N-terminal end. The tail is exposed and can be subjected to different modifications. The unit of DNA twists and the histone complex is called a nucleosome and the nucleosome is the basic unit of chromatin and higher order chromosome folding. In what way the nucleosomes arrange and fold into higher order structures is not yet completely known. What is known is that there are two different kinds of chromatin; euchromatin and heterochromatin and the difference between them depends on specific modifications of the histone tails e.g. methylation, acetylation and phosphorylation [3].

Euchromatin is known as the accessible part of the chromatin, the transcriptionally active part where most of the expressed genes are located. Euchromatin is also associated with methylation of lysine 4 of histone H3 [4].

Heterochromatin is known as the silent part of the chromatin. It is often associated with hypoacetylation of histones [5] and in fission yeast, *Schizosaccharomyces pombe*, *Drosophila* and mammals also with methylation of lysine 9 on histone H3 (H3 lys9) [4, 5]. Heterochromatin is thought to have the capability of regulating transcription activity. Long repetitive DNA sequences surrounding for example the centromeres and telomeres tend to have heterochromatic structure and it has been hypothesised that the reason for this is to stabilise and shelter the repetitive DNA sequence from homologous recombination [5]. Heterochromatin is also believed to have a function in for example long-range regulatory interactions [6] and chromosome segregation [5].

The initiation of heterochromatin assembly is called nucleation and it is believed to occur in different ways. It can occur by binding of a DNA binding protein to a specific sequence or silencer and this can trigger the nucleation. There are speculations about whether repetitive DNA like satellite repeats and transposons are involved in nucleation or not [5]. There is also another pathway called the RNAi pathway, which nucleates heterochromatin formation in fission yeast [7]. Once the nucleation has started, heterochromatin will spread. In *S. pombe*

methylated H3 lys9 is capable of binding protein Swi6 (homologous to HP1 in human) which in turn recruits the histone methyltransferase Clr4 and Clr4 will methylate the nearby histones and hence the heterocromatin is spread [8]. The heterochromatin spreads along the chromatin and stops at a specific boundary element flanking the heterochromatin [4]. Interestingly, different heterochromatin domains make use of different nucleation ways [9].

1.2 Nuclear architecture

The spatial organisation of the genes in the nucleus is believed to play a part in the regulation of genes. Several studies performed in mammalian cells have united scientists to believe that the chromosomes in the nucleus are distributed in different chromosome territories (CT). Different studies concerning more detailed organisation have given birth to different theories [10].

1.2.1 Distribution of chromosomes

One theory concerning the distribution of chromosomes in the nucleus is the interphase chromosome position gene-density theory. This theory was proposed when it was discovered that gene-poor chromosomes tended to be positioned in the nuclear periphery and gene-rich chromosomes towards the nuclear interior [10, 11]. This was shown to be true for human chromosomes in human lymphoblast cells and among different kinds of fibroblasts, and also for chicken and primate chromosomes. It was proposed that this distribution was due to transcriptional silencing at the nuclear periphery and transcriptional activity in the interior of the nucleus [10].

Another theory is the interphase chromosome position-size theory that sorts the position of the chromosomes according to size with the smallest chromosome in the interior and the largest at the periphery [10, 11]. This theory is supported by studies in human primary fibroblasts, different kind of porcine cells and special kinds of chicken fibroblasts and neurons. Perhaps none of these theories is completely correct and probably other factors than density and size matters [10].

1.2.2 Distinct or intermingling chromosome territories?

As mentioned above, all scientists agree that different CTs exist but if CTs are restricted to distinct areas or if they intermingle with each other is not clear. Studies have shown that transcription of genes occurs in distinct foci called transcription factories and some believe that these factories are located between the CTs and that active genes loop out from CTs to these transcription factories. The movement of genes into and out of transcription factories is believed to be a dynamic process but factors regulating this process are unknown [12].

Another study in human cells has shown that CTs intermingle with each other and interestingly also that this event strongly correlates with translocation frequencies. The transcription factories in this model are present in the intermingling area and there are transcription-dependent interactions that influence the extent of intermingling [13].

1.2.3 The nuclear periphery

In interphase, both mammals and *S. pombe* repetitive DNA sequences at the centromeres cluster together and in mitotically dividing *S. pombe* the cluster is localised near the spindle pole body (SPB, functional homolog to the centrosome in mammals) at the nuclear envelope. The telomeres containing repetitive DNA sequence cluster with each other in both budding yeast, *Saccharomyces cerevisiae*, and *S. pombe* and the clustered telomeres localise near the

nuclear periphery. In *S. cerevisiae* the telomeres are believed to be anchored by the protein Ku and Esc1. In *S. pombe* no such anchoring proteins have been discovered [14].

Factors that may be involved in localising silent chromatin (heterochromatin) to the nuclear periphery in multicellular aminals (metazoan) are nuclear lamins, a filamentous network of proteins positioned right under the nuclear envelope; nuclear envelope associated proteins, chromatin and proteins associated with chromatin, proteins in or by the nuclear envelope that interact with and bind directly or indirectly to DNA; and the proposed nuclear matrix [10, 14].

As mentioned above there is evidence that points out that actively transcribed genes are mostly positioned in the interior of the nucleus and that silent genes are positioned near the nuclear periphery, often in association with heterochromatin. This is seen in mammals, *Drosophila* and yeast [15]. There is also evidence for translocation of genes upon activation or repression. The loci of IgH and Igκ in developing B cells relocate away from nuclear periphery upon activation [16] and genes repressed by Ikaros (in mouse B lymphocytes) localise to centromeric heterochromatin [17]. An interesting study in *S. cerevisiae* uses a strain defective in forming silent chromatin in a normally silent region. The region was anchored artificially with the nuclear periphery and the reporter gene inserted in the locus was silenced [18].

1.3 This study

1.3.1 Schizosaccharomyces pombe – a model organism

S. pombe is a well known model organism. It is a unicellular yeast containing only three chromosomes. The reason why *S. pombe* was used in this study is that *S. pombe* has more similarities with humans concerning for example centromeres and the proteins involved in heterochromatin nucleation and spreading than for example the commonly used yeast *S. cerevisiae* [5, 19]. Furthermore *S. pombe* is easy to manipulate and have a short generation time, about 2 – 4 h [20].

Normally, *S. pombe* cells live as haploid cells but upon stimulation they can mate and form zygotes. If the stimulation stops, these zygotes can live as diploid cells during vegetative growth but if the stimulation is preserved, the zygotes enter meiosis and form haploid spores. In *S. pombe*, mating and meiosis can be induced by nitrogen starvation but the events are also dependent on mating pheromone signalling. There are two different mating types in *S. pombe*, h^+ and h^-, which carry different mating cassette genes and because of that they also express different mating pheromones. On the surface of h^+ cells the receptor for the mating pheromone produced by h^- cells is expressed and vice versa, hence h^+ cells can only mate with h^- cells and not with themselves. Wild type *S. pombe* cells, h^{90}, contains both mating cassettes and are able to switch mating types explaining the fact that mating and meiosis can be induced in a h^{90} strain [21]. Even if h^+ and h^- strains are unable to enter meiosis upon nitrogen starvation, gene expression in both strains could be affected by the starvation.

1.3.2 Previous work

A scan of the *S. pombe* genome made by Mata and co-workers [22] using DNA-microarrays examined the meiotic expression of 99.3% of the by then about 4 900 known and predicted genes in *S. pombe*. They found that more than 50% of the genes in the genome were somehow affected by the meiosis process. Mata and co-workers induced meiosis in diploid *S. pombe* cells by nitrogen starvation. Interestingly some of the genes, believed to be induced by nitrogen starvation, were clustered at five different positions on the chromosomes including the subtelomeric regions of chromosome I and II. Mata and co-workers did not believe that

the genes in each cluster are parts of an operon because they are transcribed in different orientations. Because of the proximity of the heterochromatin in the telomeres they proposed that these genes may be activated from silenced regions upon nitrogen starvation. Two of these regions were chosen to be studied and both are located on chromosome I.

The first region, named Subtel1, is located in the subtelomeric region of the left arm of chromosome I (21 – 200 kb) and contains 25 genes and predicted genes regulated during meiosis, six of them are induced by nitrogen starvation and expressed transiently [22] (Appendix 1). These six genes are concentrated in the region 58 – 145 kb [23]. The region from 0 – 200 kb was identified in another study in *S. pombe* to be regulated by Clr3 [24], an enzyme that deacetylates histones and is believed to be involved in heterochromatin regulation [25].

The second region, named Chr1cluster, is located in the middle of the left arm of chromosome I. It is about 22 kb in length and spans from about 1 832.45 – 1 854.45 kb and contains seven genes and predicted genes regulated during meiosis and six of them are thought to be induced by nitrogen starvation and expressed transiently [22] (Appendix 1). This cluster was not associated with heterochromatin or proximity to heterochromatin but perhaps it would show differences in localisation due to other epigenetic mechanisms. All of the chosen nitrogen starvation genes in the two clusters have a peak in their expression 1 h after the start of sexual differentiation and then the expression decreases [26].

One important thing to notice is that Mata and co-workers performed their study on diploid cells but this study was performed on haploid cells. When inducing meiosis in diploid cells by nitrogen starvation the cells enter meiosis directly, but when inducing meiosis by nitrogen starvation in a haploid cell it has to mate with another cell prior to meiosis [21]. Mata and co-workers have shown that the genes in Subtel1 and Chr1cluster clusters are upregulated within one hour after nitrogen removal in diploid cells directly entering meiosis.

1.3.3 Aim

This study aimed to visualise the localisation of two clusters containing nitrogen starvation genes, Subtel1 and Chr1cluster, in the fission yeast *S. pombe* and detect possible localisation changes between them during vegetative growth and during nitrogen starvation i.e. if the regulated genes change location in the nucleus upon activation.

1.3.4 Overall strategy

To visualise the localisation of the loci Subtel1 and Chr1cluster, the lac Operator/Repressor (*lacO*/LacR-GFP) recognition system can be used. This system was developed to be able to visualise DNA sequences *in situ* both in living and fixed cells and to study e.g. chromosome dynamics. The system is based on integrating *lacO* repeats at or near the locus to be studied and in the same cell have an endogenous expression of LacR fused to green fluorescent protein, GFP. The fusion protein LacR-GFP will bind to *lacO* and the GFP can be visualised using a fluorescence microscope and thereby label the locus. It is preferable to insert many copies of the *lacO* sequence since the GFP signal will then be stronger but too many copies increase the risk of homologous recombination. 256 repeats (~10 kb) are used [27, 28].

The *lacO*/LacR-GFP recognition system labels the locus of interest but it does not include a reference point. To be able to see where the locus is present relative to the nuclear envelope, the nuclear envelope can be visualised by tagging the nuclear pore protein Pom152 with yellow fluorescent protein, YFP [25, 29]. When the GFP signal and YFP signal can be

visualised in the same picture the distance between the locus of interest and the nuclear pore membrane can be measured and a possible difference between vegetative cells and nitrogen starved cells can be seen.

To investigate whether genes in the clusters are upregulated or not upon nitrogen starvation in haploid cells, RNA can be purified and analysed by reverse transcriptase polymerase chain reaction, RT-PCR, and polymerase chain reaction, PCR.

2 Material and methods

All yeast cells were grown and incubated at 30°C and all other steps were performed at room temperature if nothing else is described.

2.1 Media

The media used are described in Appendix 2.

2.2 Recurring procedures

2.2.1 Gel electrophoresis and gel purification

DNA and RNA were analysed by gel electrophoresis using 1% agarose gel at 50-120 V if nothing else is described. QIAquick® Gel Extraction Kit (QIAGEN®) was used to purify DNA from an excised gel slice and the purifications were verified by gel electrophoresis. GeneRuler™ 1kb DNA ladder (Fermentas) was loaded as reference in one or two wells of each gel (0.5 µg/well).

2.2.2 PCR

Polymerase chain reaction, PCR, was performed using Expand High fidelity PCR system (Roche) and dNTP Mix (10mM, Fermentas) if nothing else is described. The different PCR programs used are described in Appendix 3. When purified DNA was used as template the DNA was diluted 5-10 times.

2.2.3 DNA precipitation

The DNA was diluted with deionised water to a total volume of at least 300 µl. 30 µl (0.1 volume) 3 M sodium acetate and 750 µl (2.5 volumes) ice cold 96% ethanol were added and the mix was kept at -20°C over night. The frozen sample was centrifuged at 13 000 rpm at 4°C for 20 min. The DNA pellet was resuspended and washed in 300 µl ice cold 70% ethanol by centrifugation at 13 000 rpm at 4°C for 10 min. The pellet was dried and then resuspended in 30 µl deionised water. The DNA was analysed by gel electrophoresis. The approximate concentration was estimated by comparing the intensity of the DNA band to the known amount loaded in the ladder.

2.2.4 Colony PCR

A small amount of fresh cells (5 – 10 µl) was resuspended in 50 µl deionised water. 5 µl of the cell suspension was used in a PCR of a total volume of 50 µl. PCR was performed using the program JTAG (Appendix 3). The reaction was later successfully scaled down to a total volume of 30 µl.

2.2.5 DNA purification

Yeast cells were grown over night in 6 ml liquid YEA at 225 rpm to middle log phase ($5 \cdot 10^6$ – $1 \cdot 10^7$ cells/ml, cells counted using a Bürker chamber) and harvested. The cell pellet was washed once in 1 ml of 1.2 M D-sorbitol, 50 mM sodium citrate, 50 mM sodium phosphate and 40 mM EDTA (pH 5.6). The cell pellet was once again resuspended in 1 ml of the solution described above containing 0.25 mg (25 U) Zymolase 100T (Nordic BioSite), to break the cell walls. The mixture was incubated at 37ºC and after 10 min several spheroplasts were seen in a light microscope. To test at what extent the cell walls were affected, 10% SDS was added to a small sample and then the affected cells were lysed. Depending on the amount of the cells affected by Zymolase they were incubated for a further 5 min or not. To collect the spheroplasts the cells were centrifuged at 3 000 rpm for 5 min. The pellet was resuspended in 450 µl 5xTE and 50 µl 10% SDS and the mix was incubated at room

temperature for 5 min. 150 µl 5 M ice cold potassium acetate was added and the mixture was incubated on ice for 10 min and then centrifuged at 13 000 rpm at 4ºC for 10 min. 500 µl (1 volume) isopropanol was added to the supernatant before centrifugation at 13 000 rpm for 5 min. The pellet was resuspended in 250 µl 5xTE and 2 µl RNase A (10 mg/ml, Sigma) and incubated at 37°C for 20 min. 2 µl proteinase K (5mg/ml) and 2 µl 10% SDS was added. After 1 h incubation time at 55°C phenol/chloroform extractions was performed. 250 µl (1 volume) phenol was added and then the tube was vortexed and centrifuged at 13 000 rpm for 5 min. The top layer was carefully transferred to a new tube where 250 µl (1 volume) chloroform was added. The same treatment as phenol extraction was performed but with chloroform including vortexing and centrifugation. Once again the top layer was transferred to a new tube and then the DNA was purified by DNA precipitation. The genomic DNA was analysed by gel electrophoresis.

2.3 Strains

2.3.1 Crossed strains

All strains and their genotypes are listed in Table 1. Strains containing the lac repressor, LacR, fused to GFP at the *his7* locus under the control of the *dis1* promoter [30] were already prepared (PJ307, PJ240) [29, 31]. A strain containing YFP fused to Pom152 was also already prepared (PJ370) [29]. The strains PJ454, PJ470, PJ430, PJ441 and PJ466 were all obtained by crossing. The parents were mixed on a PMG total plate and cultivated for 1 – 3 days. Spore formation was confirmed with a light microscope. Cells and spores were incubated together with 1% NEE-154 Glusulase (from stock solution 10 000 U/ml, PerkinElmer®) for 1 – 3 days and vortexed every now and then to break cell walls of unconjugated cells and sporeasci to get only free spores. Spores were plated on YEA plates and one of the selective media plates (e.g. PMG –lys) and then the colonies were replica plated to several selective media plates to find colonies with the desired genotype. Colonies of interest were picked and streaked on YEA plates for single colonies and single colonies were picked and patched on YEA plates. Patches were replica plated once again to several selective media plates to verify their genotype.

2.3.2 Transformed strains

The strains PJ488, PJ489, PJ487, PJ484, PJ502B, PJ494, PJ492 and PJ493 were obtained by transformation by electroporation of PJ430, PJ454, PJ466 and PJ470. The strains PJ522, PJ525, PJ521, PJ519, PJ526, PJ513, PJ511 and PJ512 were obtained by chemical transformation of PJ488, PJ489, PJ487, PJ484, PJ502B, PJ494, PJ492 and PJ493. Transformations are described below.

Table 1. PJ240, PJ138, PJ307, PJ139 and PJ370 are the origins to PJ454, PJ470, PJ430, PJ441 and PJ 466, obtained by crossing. PJ488, PJ489, PJ487 and PJ484 are transformed strains of PJ430 and PJ454 containing *lacR-GFP* and *ura4C*. PJ502B, PJ494, PJ492 and PJ493 are transformed strains of PJ466 and PJ470 containing both *lacR-GFP*, *ura4C* and *pom152-YFP*. PJ522, PJ525, PJ521 and PJ519 are transformed strains of PJ488, PJ489, PJ487 and PJ484 containing both *lacR-GFP* and *lacO*. PJ526, PJ513, PJ511 and PJ512 are transformed strains of PJ502B, PJ494, PJ492 and PJ493 containing *lacR-GFP*, *pom152-YFP* and *lacO*. PJ508 is the product of crossing PJ370 and PJ519 containging *lacR-GFP*, *pom152-YFP* and *lacO*.

Strains	Genotype	Source
CT2 121-4 = PJ240	*h^{90} his7^{+}::dis1placR-GFP ade3*[*::kanMX6 ura4^{+} lacO*] *lys1-131 leu1-32 ura4-D18 ade6-M216*	[31]
PJ138	*h^{+} his7-366 leu1-32 ura4-D18 ade6-M210*	Lab stock
PJ454	*h^{90} his7^{+}::dis1placR-GFP leu1-32 ura4-D18 ade6-M210*	This study
PJ370	*h^{+} his7-366 pom152-YFP::kanMX6 lys1- leu1-32 ura4-D18 ade6-DN/N*	[29]
PJ470	*h^{90} his7^{+}::dis1placR-GFP pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-M210*	This study
PJ307	*h^{+} his7^{+}::dis1placR-GFP cut12-CFP::kanMX6 lys1^{-} leu1-32 ura4-D18 ade6-DN/N*	[29]
PJ139	*h^{-} his7-366 leu1-32 ura4-D18 ade6-M216*	Lab stock
PJ430	*h^{+} his7^{+}::dis1placR-GFP leu1-32 ura4-D18 ade6-*	This study
PJ441	*h^{-} his7-366 pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-*	This study
PJ466	*h^{+} his7^{+}::lacR-GFP pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-*	This study
PJ488	*h^{+} his7^{+}::dis1placR-GFP leu1-32 ura4-D18 ade6^{-} subtel1*::[*ura4C hphMX6*]	This study
PJ489	*h^{+} his7^{+}::dis1placR-GFP leu1-32 ura4-D18 ade6^{-} chr1cluster*::[*ura4C hphMX6*]	This study
PJ487	*h^{90} his7^{+}::dis1placR-GFP leu1-32 ura4-D18 ade6-M210 subtel1*::[*ura4C hphMX6*]	This study
PJ 484	*h^{90} his7^{+}::dis1placR-GFP leu1-32 ura4-D18 ade6-M210 chr1cluster*::[*ura4C hphMX6*]	This study
PJ502B	*h^{+} his7^{+}::dis1placR-GFP pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-subtel1*::[*ura4C hphMX6*]	This study
PJ494	*h^{+} his7^{+}::dis1placR-GFP pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-chr1cluster*::[*ura4C hphMX6*]	This study
PJ492	*h^{90} his7^{+}::dis1placR-GFP pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-M210 subtel1*::[*ura4C hphMX6*]	This study
PJ493	*h^{90} his7^{+}::dis1placR-GFP pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-M210 chr1cluster*::[*ura4C hphMX6*]	This study
PJ522	*h^{+} his7^{+}::dis1placR-GFP leu1-32 ura4-D18 ade6^{-} subtel1*::[*ura4 hphMX6 ura4C lacO*]	This study
PJ525	*h^{+} his7^{+}::dis1placR-GFP leu1-32 ura4-D18 ade6^{-} chr1cluster*::[*ura4 hphMX6 ura4C lacO*]	This study
PJ521	*h^{90} his7^{+}::dis1placR-GFP leu1-32 ura4-D18 ade6-M210 subtel1*::[*ura4 hphMX6 ura4C lacO*]	This study
PJ519	*h^{90} his7^{+}::dis1placR-GFP leu1-32 ura4-D18 ade6-M210 chr1cluster*::[*ura4 hphMX6 ura4C lacO*]	This study
PJ526	*h^{+} his7^{+}::dis1placR-GFP pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-subtel1*::[*ura4 hphMX6 ura4C lacO*]	This study
PJ513	*h^{+} his7^{+}::dis1placR-GFP pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-chr1cluster*::[*ura4 hphMX6 ura4C lacO*]	This study
PJ511	*h^{90} his7^{+}::dis1placR-GFP pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-M210 subtel1*::[*ura4 hphMX6 ura4C lacO*]	This study
PJ512	*h^{90} his7^{+}::dis1placR-GFP pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-M210 chr1cluster*::[*ura4 hphMX6 ura4C lacO*]	This study
PJ508	*h^{90} his7^{+}::dis1placR-GFP pom152-YFP::kanMX6 leu1-32 ura4-D18 ade6-M210 chr1cluster*::[*ura4 hphMX6 ura4C lacO*]	This study

2.4 Integration of *lacO* repeats

The integration of *lacO* repeats in the *S. pombe* genome was performed in two steps as described previously, with the exception that a hygromycin B resistance was used instead of the G418 resistance gene [32, 33]. First only a part of the *ura4*$^+$ gene, the *ura4C* gene, and the hygromycin B resistance gene were transformed into the genome by electroporation using homology to the *S. pombe* genome. Then the whole *ura4*$^+$ gene and *lacO* repeats were inserted by chemical transformation using homology to the *ura4C* sequence. This is also described in Figure 1.

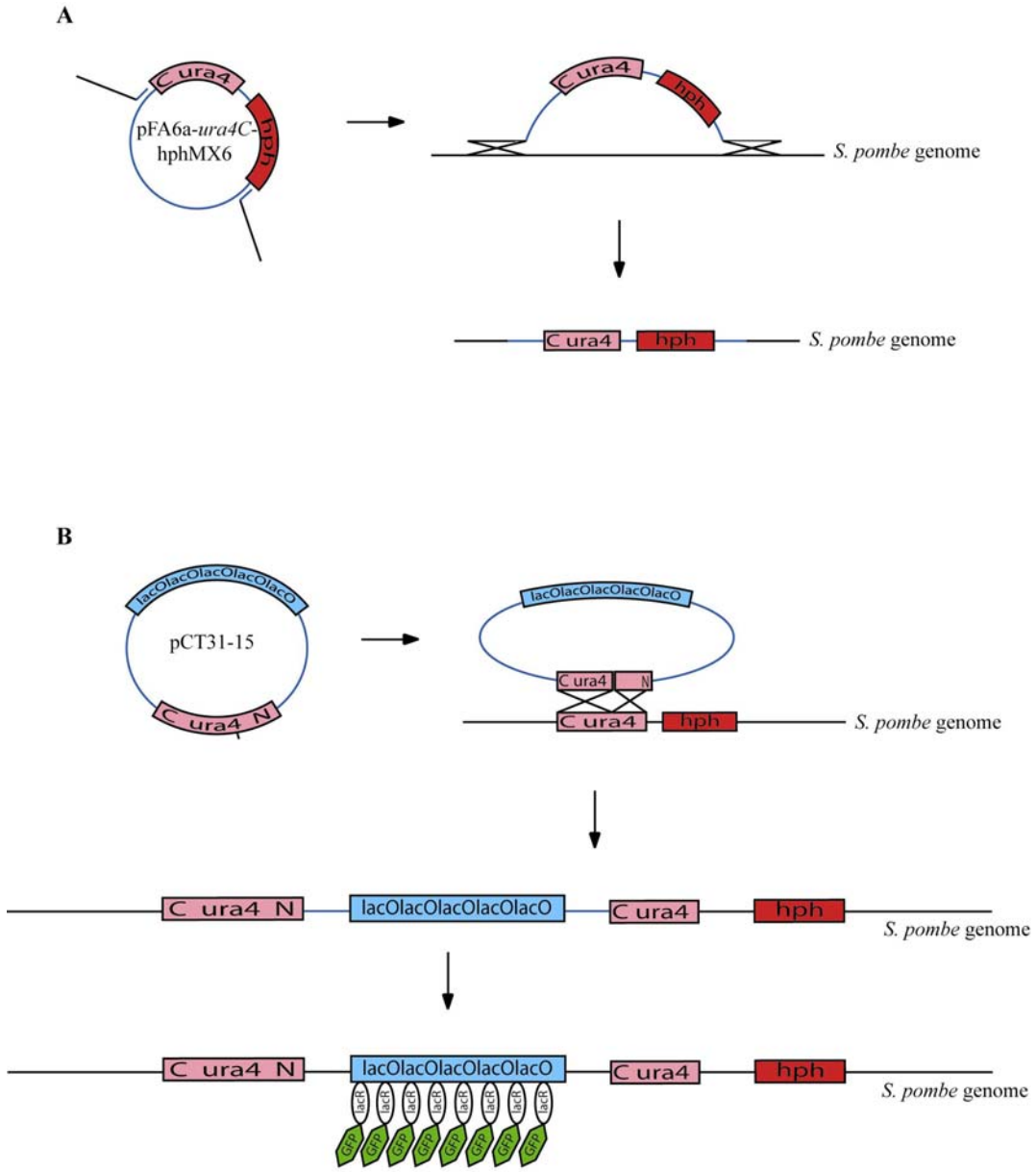

Figure 1. Overview for the strategy used to integrate *lacO* repeats near the clustered genes. **A:** The *ura4C* gene was inserted in the *S. pombe* genome by homologous recombination. The DNA used for transformation was created in a PCR reaction with pFA6a-*ura4C*-hphMX6 as template and long primers with homology to the genome. **B:** The *lacO* repeats was inserted in the *S. pombe* genome with the linearised plasmid pCT31-15 by homologous recombination to the *ura4C* gene inserted in **A**. The location where the *lacO* repeats was inserted was visualised via the LacR-GFP expressed in the same cell bound to *lacO*.

2.4.1 Construction of plasmid pFA6a-ura4C-hphMX6
A TOPO® 2.1 vector with a part of the *S. pombe ura4* gene inserted (truncated at the N-termial end) was already prepared [33]. The *ura4C* fragment inserted in the TOPO® 2.1 vector was obtained by a PCR using primer pair ura4 C-term:1/ura4 C-term:2 (Appendix 4) containing restriction sites for *Bam*HI. *ura4C* was cut out with *Bam*HI (Fermentas). Three reactions (20 µl each) were prepared and incubated at 37ºC for 3 h.

A vector containing hygromycin B resistance, pFA6a-hphMX6 (Euroscarf Van Driessche-SET, [34]), was linearised by cutting with restriction enzyme *Bgl*II (Fermentas). Two reactions (20 µl each) were prepared and incubated at 37ºC for 3 h.

The similar reaction mixtures described above were pooled and analysed by gel electrophoresis and the vector and the insert were excised and purified from the gel.

The purified linearised vector pFA6a-hphMX6, was treated with the enzyme SAP (Shrimp Alkaline Phosphatase) (Fermentas) to dephosphorylate the 5' end of the vector and to avoid self ligation. The treatment was performed according to Fermentas protocol (Protocol for Dephosphorylation of DNA 5'-termini) using 25 µl (~ 0.6 µg) purified vector and the reaction was stopped by heating at 65ºC for 10 min.

The insert, the *ura4C* gene, was ligated into the pFA6a-hphMX6 vector with T4 DNA Ligase. Appropriate amounts of *ura4C* DNA and vector DNA were mixed with 2 µl 10x Buffer for T4 DNA Ligase, 1 µl (5 Weiss U) T4 DNA ligase (reagents from Fermentas) and deionised water to a total volume of 20 µl and incubated at 16ºC over night.

The ligations were then transformed into *E. coli* SF8 cells by heat shock (42ºC for 40 s) using 5 µl ligation mixture to 50 µl cells. After the heat shock 500 µl LB medium was added to each reaction and the mixtures were incubated at 37ºC for 30 min at 200 rpm before spreading on LB plates with 150 mg/l ampicillin. The plates were grown at 37ºC over night. Colonies were picked and cultivated in 3 ml liquid LB with 150 mg/l ampicillin at 37 ºC at 200 rpm over night. The plasmids were purified using QIAprep® Spin Miniprep Kit (QIAGEN). To verify the integration of the insert, the purified plasmids were digested with *Eco*RI and *Hin*DIII (Fermentas). The reaction mixtures were incubated at 37ºC for 1.5 h and then analysed by gel electrophoresis using a 0.8% agarose gel. Two clones with the right restriction fragments (2.4 kb, 1.5 kb, 1.0 kb) were chosen and cultivated in 25 ml liquid LB with 150 mg/l ampicillin at 37ºC at 200 rpm over night. The plasmids were purified using E.Z.N.A.® Plasmid Midiprep Kit (E.Z.N.A.®) and eluated in 600 µl deionised water. The new plasmid was named pFA6a-*ura4C*-hphMX6.

To create DNA for the electroporation, the PCR method described by Bähler and colleagues [35] was performed. This technique is based on the use of primers complementary to pFA6a-*ura4C*-hphMX6 and tagged with a long sequence (100 bp) with homology near the loci of the *S. pombe* genome of interest (~3 kb from the Chr1cluster locus and ~17 kb from the Subtel1 locus). The PCR was performed with the primer pairs Subtel1F/R and Chr1clusterF/R (Appendix 4). The program JTAG (Appendix 3) was run and several reactions of each primer pair were performed (50 µl each). The reactions were pooled the PCR products were purified by DNA precipitation.

2.4.2 Electroporation
Yeast cells were cultivated over night in 50 ml liquid YEA at 225 rpm and when the cells were in middle log phase, approximately 10^9 cells were harvested per strain by centrifuging at 3 600 rpm at 4ºC for 5 min. Cells were washed three times with 20 ml ice cold 1.2 M D-sorbitol (Sigma) at 4ºC. Cells were resuspended in 1.2 M ice cold D-sorbitol to a final volume of 1 ml and then 200 µl (~ $2 \cdot 10^8$ cells) of cells were aliquoted into one eppendorf tube each. 150 ng – 500 ng desalinated DNA (the product from the pFA6a-*ura4C*-hphMX6 PCR) was added to the cells prior the electroporation. The electroporation was performed with Gene pulser® II (Biorad) and the settings were 2.25 kV, 200 Ω, 25 µF. Directly after the electric pulse 300 µl 1.2 M ice cold D-sorbitol was added. The cells were carefully centrifuged at 4 000 rpm for 30 s and 200 – 300 µl of the supernatant was discarded. The cells were resuspended in the remaining supernatant and plated on YEA plates. The transformants were grown over night to allow the expression of the hygromycin B resistance gene to start and then they were replicated onto freshly prepared YEA plates with 200 mg/l hygromycin. Single colonies were picked and streaked for new single colonies on YEA plates with 100 mg/l hygromycin. To confirm stable transformant, single colonies from that plate were picked and patched several times on YEA plates and finally replica plated onto YEA plates with 200 mg/l hygromycin.

To screen and verify the transformants and to decide in which direction the *ura4C* insertion was made (i.e. in which direction *ura4C* was cloned into the pFA6a-hphMX6 vector), colony PCR was performed. Primer pairs used were CtrlSubtel1F/R, CtrlChr1clusterF/R which all bind on the genomic DNA outside the transformed insertion; CtrlSubtel1F/Ura4R, CtrlSubtel1R/Ura4R, CtrlChr1clusterF/Ura4R, CtrlChr1clusterR/Ura4R of which one part of the pair binds outside and the other one binds inside of the transformed insertion. To confirm the right transformants from the electroporation and due to problems concerning the colony PCR, genomic DNA was purified and an ordinary PCR (JPCR, Appendix 3) was performed using using primer pairs CtrlSubtel1F/R, CtrlSubtel1F/Ura4R, CtrlSubtel1R/Ura4R, CtrlChr1clusterF/R, CtrlChr1clusterF/Ura4R and CtrlChr1clusterR/Ura4R. The PCR products were analysed by gel electrophoresis.

2.4.3 lacO repeats for transformation
The vector pCT31-15 is a pBluescript®II KS(+) plasmid with a 10 kb long *lacO* repeat array (256 repeats) and the full length *ura4*$^+$ gene (including the N-terminal). The vector was kindly provided by A. Yamamoto (Kansai Advanced Research Center, Kobe). To linearise the vector it was cut with restriction enzyme *StuI* (New England BioLabs®) that only cut the vector at one position and that was in the *ura4*$^+$ gene. The mix of a total volume of 20 µl was incubated at 37°C for 3.5 h. Cleaved vector was analysed by gel electrophoresis and the DNA concentration was estimated by comparing the intensity of the cleaved vector to that of the known amount loaded ladder.

2.4.4 Chemical transformation
Yeast cells were grown over night in liquid YEA at 225 rpm to log phase then harvested by centrifugation at 3 000 rpm for 5 min and after that washed once in 25 ml deionised water. The pellet was resuspended in deionised water to a final volume of 1 ml and 100 µl of cells were then aliquoted into one eppendorf tube each. The cells were centrifuged at 13 000 rpm for 30 s and the supernatant was discarded. 360 µl of the transformation mixture was added and the pellet was resuspended. The transformation mixture consisted of 240 µl PEG 3000 50% w/v (Fluka), 36 µl 1 M lithium acetate, 50 µl boiled salmon sperm-carrier DNA (Sigma) and 34 µl DNA and deionised water. The transformation and cell mixture was incubated at

42°C for 40 min and then centrifuged at 13 000 rpm for 30 s. The pellet was resuspended in deionised water to a total volume of 200 µl. Finally the cells were spread on PMG –ura plates. About $5 \cdot 10^7 – 10^8$ cells were used for one transformation and about 40 – 320 ng DNA was used for one transformation. Transformants were picked and streaked for single colonies on PMG –ura plates. Single colonies were picked and patched several times on YEA plates and finally the patches were replica plated on PMG –ura plates to confirm stable transformants. The transformants were verified by colony PCR using primer pairs CtrlSubtel1F/Ura4F and CtrlChr1clusterF/Ura4F and by seeing a clear GFP-signal in a fluorescence microscope.

2.5 Induction of nitrogen starvation genes in Chr1cluster

2.5.1 Nitrogen starvation

This experiment was only performed on strains PJ508, PJ512 and PJ513. In all the three strains the *lacO* repeats was inserted near Chr1cluster. PJ508 and PJ512 have the same genotype, but were obtained independently. PJ508 and PJ512 are h^{90} strains and nitrogen starvation will lead to meiosis in these strains. PJ513 on the other hand is a h^+ strain and without mating partner nitrogen starvation cannot induce meiosis.

Yeast cells were grown over night in liquid EMM –ura (with sterile filtered glucose which reduces autofluorescence in the microscope) at 225 rpm to log phase. The cells were split in two samples and harvested by centrifugation at 3 600 rpm for 5 min. One sample of the cells was washed three times with 20 ml EMM –ura and the other sample was washed three times with 20 ml EMM –ura $–NH_4Cl$. The cells were resuspended in a volume of the same media they had been washed in, to a final concentration of about $4 \cdot 10^6$ cells/ml. The cells were grown at 30°C at 225 rpm and samples (~ $4 \cdot 10^7$ cells) were taken every 20^{th} min. Two experiments were performed, experiment 1 and experiment 2. Experiment 1 was performed on PJ508 (h^{90}) and samples were taken directly after the wash (t=0), after 20, 40, 60, 80, 100 and 120 min. Experiment 2 was performed on PJ512 (h^{90}), PJ513 (h^+) and samples were taken before the wash, directly after the wash (t=0) and after 20, 40, 60, 80, 100, 120, 140, 160 min.

2.5.2 RNA purification

To investigate if the genes in the loci were upregulated during nitrogen starvation, RNA was purified from the samples and analysed by RT-PCR and PCR. RNA was purified using RNeasy® Mini Kit (QIAGEN®) using 0.5mg/ml (50 U/ml) Zymolase 100T (Nordic BioSite) in Buffer Y1. Before the last centrifuging step, the columns were incubated for 1 min with 30 µl RNase-free water.

2.5.3 DNase treatment

In experiment 1, DNase I from the kit RiboPure™ Yeast (Ambion) was used. 10 µl RNA sample, 1 µl 10x DNase I Buffer and 2 µl (4 U) DNase I was mixed and incubated at 37°C for 30 min. The reaction was stopped when 1.3 µl DNase Inactivation Reagent was added and the mixture was vortexed and incubated at room temperature for 5 min. To remove the DNase Inactivation Reagent the mix was centrifuged at 13 000 rpm for 2 min and the supernatant containing RNA was transferred to a fresh tube.

In experiment 2, DNase I from Fermentas was used (1 U/µl) and the RNA was treated according to the protocol of the manufacturer (Protocol for Preparation of DNA-free RNA prior to RT-PCR). Using this DNase I kit, the RNA was more diluted. The DNase treated RNA samples from both experiments were analysed by electrophoresis to investigate the quality of the RNA.

2.5.4 RNA concentration
The RNA concentration was obtained by measuring A_{260} in a GeneQuant*pro* spectrophotometer (Amersham).

2.5.5 Reverse transcription PCR
Reverse transcription PCR, RT-PCR, was performed to produce cDNA of the DNase treated RNA. To be able to quantify the RNA concentration of chosen genes, equal amounts of RNA was used in all samples in the same experiment. At the first experiment, 1 µg RNA/reaction was used but at the second experiment only 144 ng RNA/reaction was used. The reaction was performed with RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas) according to the protocol of the manufacturer (Protocol for First-strand cDNA Synthesis) using total RNA and Oligo $(dT)_{18}$ primer. For each sample, a duplicate was made containing all of the reagents except reverse transcriptase. If the RNA samples were contaminated by DNA it would be detected in the duplicates in the following PCR reaction.

2.5.6 Quantitative PCR after RT-PCR
To verify that the genes in the Chr1cluster were upregulated, primers for two of the genes in this cluster (*fur4* and *SPAC1399.01c*) were chosen (primer pairs Chr1clusterMF/R (*fur4*) and Chr1clusterSF/R (*SPAC1399.01c*), Appendix 4). *fur4* is located in the middle of the Chr1cluster cluster and *SPAC1399.01c* is located at one end of the cluster, but not towards the same side that the *lacO* repeats was inserted. As a positive control, primers for the gene *ste11* were used (Appendix 4). *ste11* is positioned on chromosome II [23] and it is known to be upregulated in both h^{90} and h^{+} cells during nitrogen starvation [21]. To estimate the expression of chosen genes, 1 µl cDNA from RT-PCR was used as template for PCR with *Taq* DNA Polymerase (native, with BSA, Fermentas). Two PCR with different number of cycles were performed for both experiments; JPCR21 (21 cycles) and JPCR23 (23 cycles) (Appendix 3). The reason why two different PCR program were performed was to find the optimal cycle number to see a difference between the amount of products and hence the amount of RNA (to compare the quantity of transcribed genes). PCR was performed with the duplicates from the RT-PCR without reverse transcriptase as template using only primers Ste11F/R. The total volume of each reaction (10 µl) was analysed by gel electrophoresis.

2.5.7 Microscopy
To investigate where in the cell nucleus the Chr1cluster was located, the cells were observed in a fluorescent microscope. This was performed on strains PJ512 and PJ513. Cells were cultivated and washed as described above (nitrogen starvation) but in a smaller volume. To reduce autofluorescence, it was very important to grow the cells in media containing filter sterilised glucose instead of autoclaved glucose. Samples (500 µl) were taken directly after the wash (t=0), after 70 min and after 170 min. The reason why samples were taken after 70 and 170 min (and not after 80 and 180 min) was that it took about 10 min to prepare the cells for the microscope. The cells were centrifuged at 8 000 rpm for 2 min and more than half of the supernatant was discarded and the cells were resuspended in the rest of the supernatant. 7.5 µl of the cell suspension was mixed with 7.5 µl lectin (1 mg/ml, Sigma) to fix the cells on a cover slip. 7.5 µl media was set on a microscope slide and covered with the cover slip containing the cells and sealed with nail varnish. GFP was excited using a mercury lamp that emitted a spectrum of wavelengths. The cells were observed in a fluorescence microscope using a GFP filter and pictures were taken. The exposure time was 500 ms and 12 slides 0.258 µm thick were taken using AxioCam MRm (Zeiss) and AxioVision 4.5 software (Zeiss). The slides (z-stacks) were exposed to digital deconvolution and 3D images were created.

3 Results

In all gel photos, the letter L means that GeneRuler™ 1 kb DNA Ladder (Fermentas) was loaded in the well.

3.1 Strains

Crossed and transformed strains are listed in Table1.

3.2 Construction of plasmid pFA6a-*ura4C*-hphMX6

Figure 2 shows a gel picture of the DNA to be used for electroporation. It was produced by PCR using the long primers with homology to the *S. pombe* genome. The concentrations were approximated by roughly estimating the bands in lanes 3 and 6 (20 times dilution of the DNA) to about as strong as the 2.5 kb band (known amount 33.5 ng) and that resulted in a DNA concentration of about $33.5 \cdot 20 = 670$ ng/µl. The products should be about 2.8 kb in length and that is exactly right although the bands in lane 1 and 4 (undiluted) look a bit shorter but that is probably due to the overloading of the wells.

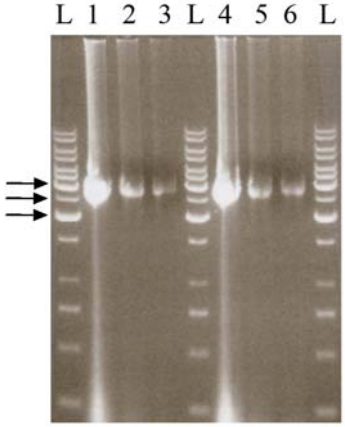

Figure 2. Gel photo of precipitated DNA for the *ura4C* transformation. Lane 1, 2, 3 are different volumes (1 µl, 0.1 µl, 0.05 µl) of PCR product from Subtel1F/R and lane 4, 5, 6 are different volumes (1 µl, 0.1 µl, 0.05 µl) of PCR product from Chr1clusterF/R. The arrows correspond to from the top; 3.0 kb, 2.5 kb and 2.0 kb.

3.3 Verification of transformants and the direction of the *ura4C* insertion

Colonies from both electroporation and chemical transformation were verified with colony PCR. Figure 3A shows an example of gel pictures from colony PCR (electroporated transformants) analysed by gel electrophoresis. Lanes 1 and 2 are negative controls and should be blank while lanes 3, 4, 5, 6 are positive controls. Figure 3B shows the primers and PCR products if the transformation has not succeeded, and Figure 3C if it has succeeded with a DNA insert at the right locus. In this experiment transformants analysed in wells 34-38 and 44 are correct ones (transformant in well 37 was used in further work) while those analysed in wells 8, 9, 11, 14-18, 20-22, 33, 39, 41 are wrong ones. No product at all indicates neither that the transformant is wrong nor that it is right, but probably that the PCR did not work. To solve this problem PCR was performed with purified DNA as template. Purified DNA enhances the chances to receive long PCR products.

To examine in which direction *ura4C* was inserted in the genome (i.e. in which direction *ura4C* was cloned into the pFA6a-hphMX6 vector), a PCR with an internal primer (Ura4R) and one external primer was performed with the transformed genome as template (both cells and purified DNA were used as template). An example of the products can be seen in Figure 4. Lane 1 shows a PCR product of about 2.6 kb in length and this is the product from primer pair CtrlChr1clusterR/Ura4R. Lane 2 does not show any band at all from primer pair CtrlChr1clusterF/Ura4R and lane 3 shows PCR product of about 3.0 kb in length from primer

pair CtrlChr1clusterF/R. The length is difficult to decide because of the high intensity of the band. The product in lane 3 confirms that the insert is at the right locus. The product in lane 1 and the absence of product in lane 2 reveal that the *ura4C* was inserted in the genome transcribed away from the hygromycin gene and towards the forward primer on the genome (Figure 4B). Since the DNA used for electroporation originated from the same clone of pFA6a-*ura4C*hphMX6 plasmid, all of the tranformants contained *ura4C* in the same direction and this was confirmed for all the transformants.

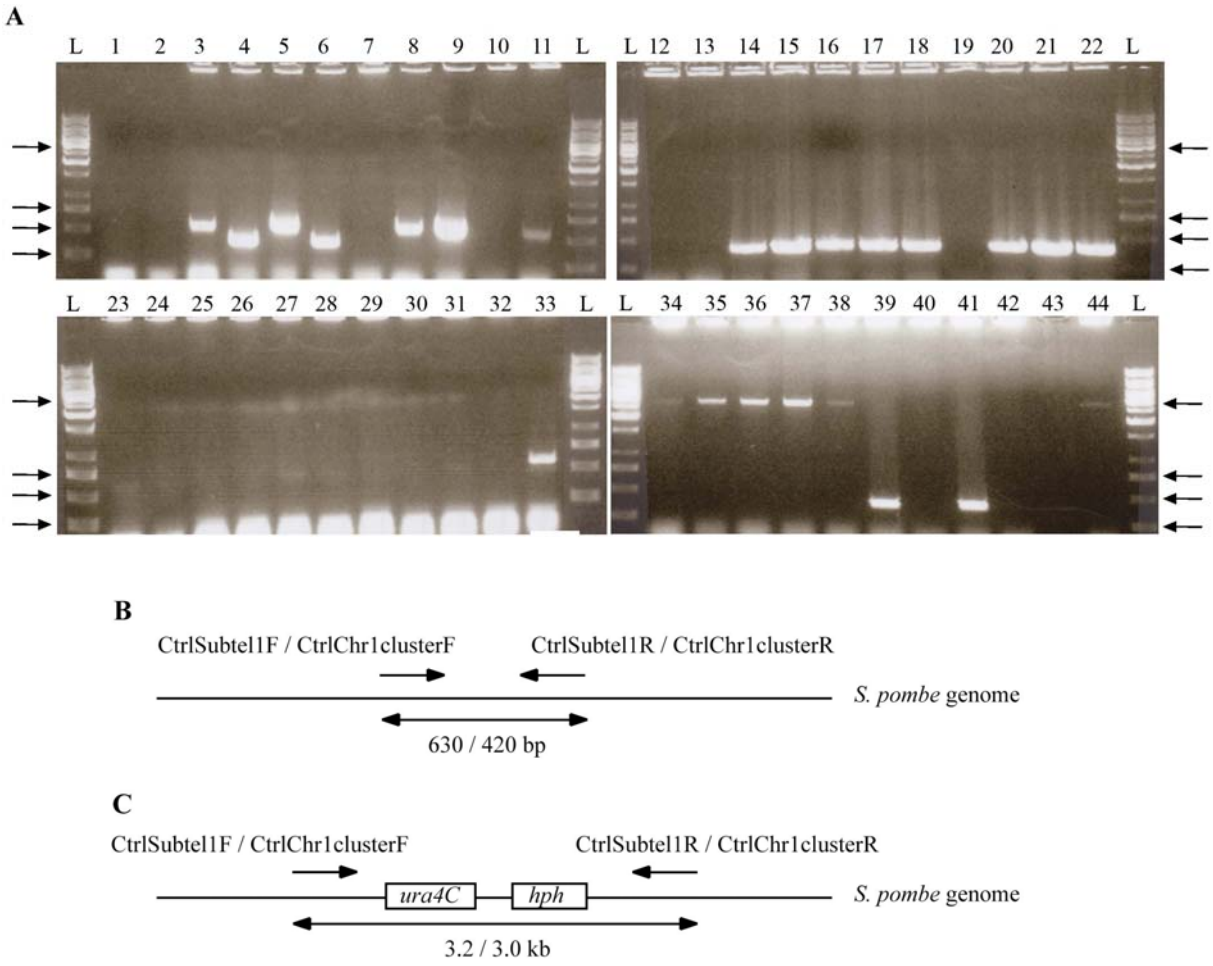

Figure 3. A: Gel photos of products from colony PCR on cells transformed by electroporation. Lanes 1 and 2 are negative controls with no DNA template using primer pairs CtrlSubtel1F/R (lane 1) and CtrlChr1clusterF/R (lane 2) and these lanes should be blank. Lanes 3-6 are positive controls with DNA from PJ430 (not transformed) in lanes 3 and 4, and DNA from PJ454 in lanes 5 and 6. In lanes 3 and 5 CtrlSubtel1F/R have been used and these lanes show products of 630 bp in length (see **B**) and in lanes 4 and 6 CtrlChr1clusterF/R have been used and these lanes show products of 420 bp (see **B**). Lanes 7-13 show PCR products from colony PCR with CtrlSubtelF/R on PJ430 cells transformed with *ura4C* and hph at Subtel1. Lanes 23-33 show PCR products from colony PCR with CtrlSubtelF/R on PJ454 cells transformed with *ura4C* and hph at Subtel1. Lanes 14-22 show PCR products from colony PCR with CtrlChr1clusterF/R on PJ430 cells transformed with *ura4C* and hph at Crh1cluster. Lanes 34-44 show PCR products from colony PCR with CtrlChr1clusterF/R on PJ454 cells transformed with *ura4C* and hph at Crh1cluster. The arrows correspond to from the top; 3.0 kb, 750 bp, 500 bp and 250 bp. **B**: PCR products if DNA has not been transformed into the right locus. **C**: PCR products if DNA has been transformed into the right locus. The figures are not to scale.

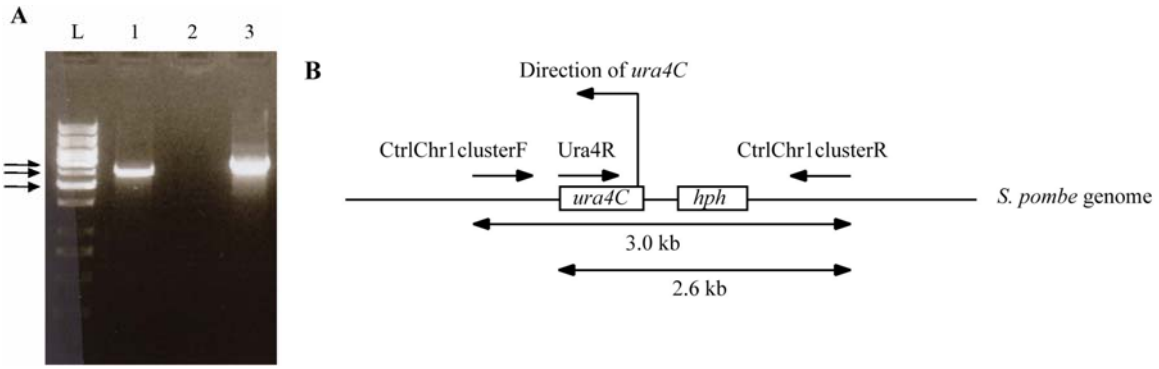

Figure 4. A:Gel photo of PCR products using purified DNA from PJ454 as template in the PCR. PJ454 was transformed by chemical transformation with *ura4* and *lacO* repeats as template in the PCR. Lane 1 shows the PCR product using primers CtrlChr1clusterR and Ura4R. Lane 2 shows the PCR product using primers CtrlChr1clusterF and Ura4R and lane 3 shows the PCR product using primers CtrlChr1clusterF/R. The arrows correspond to from the top; 3.0 kb, 2.5 kb and 2.0 kb. **B**: The primers, the lengths of the PCR products and the direction of *ura4C* are shown. The figure is not to scale.

To verify that the *lacO* repeats transformed by chemical transformation were integrated at the right locus, colony PCR was performed. A primer, Ura4F, that binds to the N-terminal of *ura4*$^{+}$ was used together with an external primer (e.g. CtrlSubtel1F or CtrlChr1clusterF). Since the direction of the *ura4C* insertion was known those were the primers to be used. A gel picture is shown in Figure 5. Lanes 1, 3, 5, 6, 7, 8 shows the correct product of 1.1 kb. Transformants yielding bands in lanes 3 and 5 were used in further work.

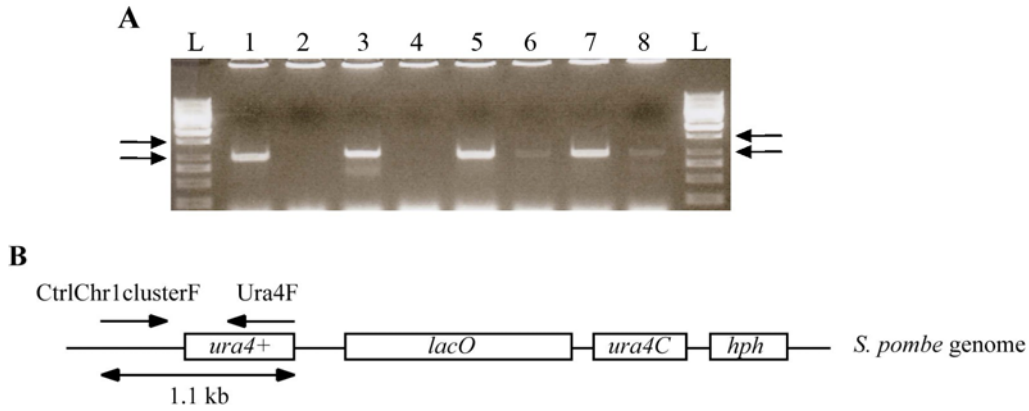

Figure 5. A: Gel photo of products from colony PCR on cells chemically transformed with linearised pCT31-15. Lane 1-3 shows products from colony PCR using chemically transformed PJ493. Lane 4 shows the product from colony PCR using nontransformed PJ494 and lane 5-8 show PCR products from colony PCR on chemically transformed PJ494. Primers CtrlChr1clusterF and Ura4F were used in all PCRs. The arrows correspond to from the top; 1.5 kb and 1.0 kb. **B**: Primer binding sites and PCR product are shown. The figure is not to scale.

3.4 RNA purification and DNase treatment

A chosen gel photo of DNase I treated purified RNA from experiment 2 is shown in Figure 6. 1 µl of each sample was loaded and gave rise to two distinct band of ribosomal RNA on the gel. These RNA bands were seen in all of the gels. The purified RNA in experiment 1 was very concentrated but the RNA in experiment 2 was more diluted. This dilution was due to the different methods the RNA was DNase I treated with.

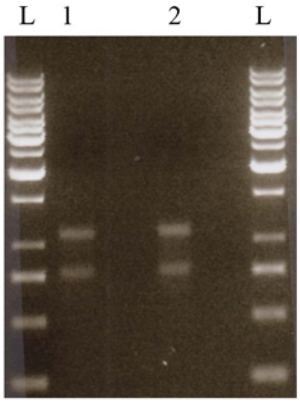

Figure 6. Gel photo of DNase I treated RNA purified from the samples taken in experiment 2. In lane 1 RNA from PJ512 before wash is seen, and in lane 2 RNA from PJ513 before wash can be seen.

3.5 RNA concentration

Original data from the RNA concentrations measured by a spectrophotometer are listed in Appendix 5.

3.6 Quantitative PCR after RT-PCR

Figure 7, 8 and 9 shows gel pictures of the samples from the quantitative PCR performed after the RT-PCR. In Figure 7 the PCR products from the duplicates made in the RT-PCR without reverse transcriptase are shown. No products are visible and this confirms that the samples were DNA free. In Figure 8 the PCR products from the PCR after RT-PCR from experiment 1 are shown and Figure 9 shows the same thing for experiment 2. Only the results from the PCR run with 21 cycles are shown here, at that number of cycles gave the highest resolution.

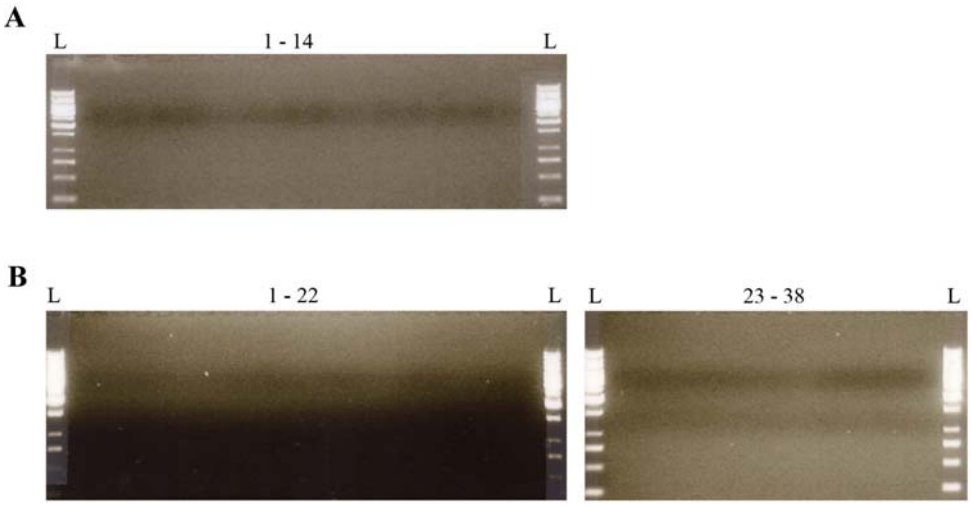

Figure 7. Gel photos analysing PCR products from PCR with the duplicates without reverse transcriptase in the RT-PCR. **A** shows the 14 samples from experiment 1 and **B** shows the 38 samples from experiment 2. No products can be seen which means that the RNA was not contaminated by DNA.

3.7.1 Experiment 1

In Figure 8 the gels from experiment 1 shows a clear increase of PCR products in the nitrogen starved cells of PJ508 (h^{90}) within 40 min. It is difficult to compare different gel pictures because of the difference in brightness and differences in ethidium bromide staining of the gel. But the amount of ladder loaded was always the same and could therefore be used for reference. One possible way to better estimate the quantity is to use software (e.g.Quantity One).

There is no clear decrease of the amount of PCR products but after 80 min the expression reached a plateau. There is a possible slight increase of the PCR products even in the samples not exposed to nitrogen starvation. This increase was perhaps due to that the cells reached late log phase. To summarise experiment 1, there was a clear upregulation of *ste11*, *fur4* and *SPAC1399.01c* within 40 min after nitrogen removal and using our quantification method the expression reached a plateau after 80 min.

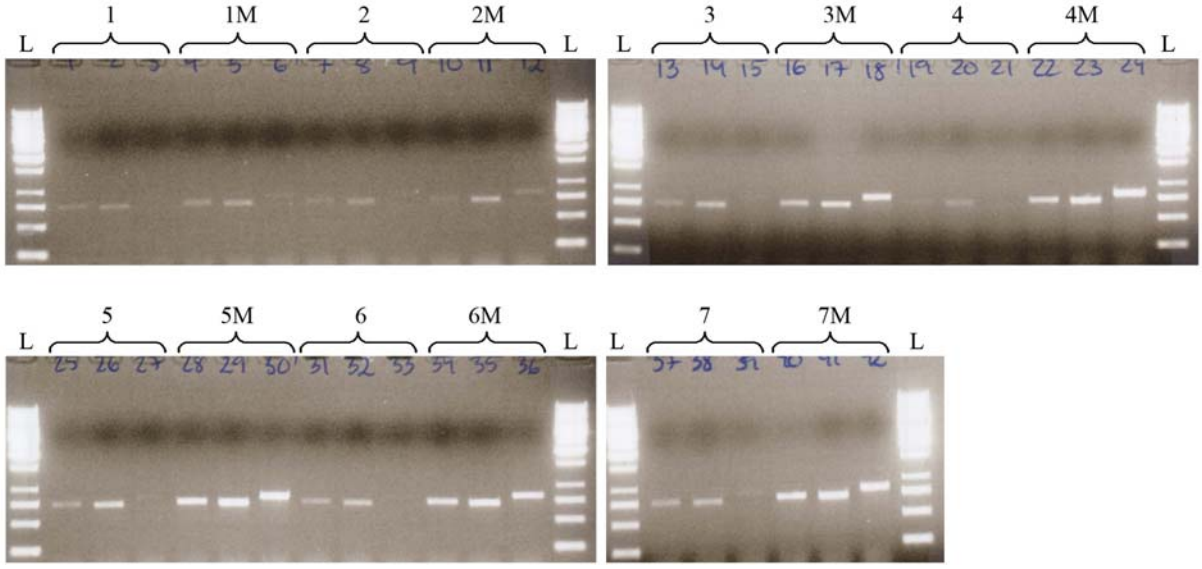

Figure 8. Gel photos showing PCR products from the PCR after RT-PCR in experiment 1 on PJ508 to quantify the expression of the genes *ste11*, *fur4* and *SPAC1399.01c*. M mean cells grown in EMM –ura –NH_4Cl and no M means cells grown in EMM –ura. 1 means samples taken at t=0 (nitrogen starvation start), 2 at t=20 min, 3 at t=40 min, 4 at t=60 min, 5 at t=80 min, 6 at t=100 min and 7 at t=120 min. The three lanes next to each other corresponds to PCR using three different set of primers: Ste11F/R (*ste11*), Chr1clusterMF/R (*fur4*) and Chr1clusterSF/R (*SPAC1399.01c*). A clear upregulation of all the three genes can be seen during nitrogen starvation.

3.7.2 Experiment 2

In this experiment nitrogen starvation was performed on PJ512 (h^{90}) and PJ513 (h^{+}) cells. To investigate the expression of the three genes before the wash, and to notice a possible decrease, samples were taken at three further time points; before the wash, 140 min after induction start and 160 min after induction start. In Figure 9 the gel pictures from experiment 2 are shown. No noticeable increase can be seen in the samples not exposed to nitrogen starvation, neither in PJ512 nor in PJ513. There is a clear increase of the expression of the three genes in the induced samples within 40 min both in PJ512 and PJ513, and a plateau is reached after 80 – 100 min. There is no difference in the induction of the three genes when comparing the h^{90} (PJ512) and h^{+} (PJ513) strains. No noticeable decrease is seen. To summarise experiment 2, there was an upregulation of *ste11*, *fur4* and *SPAC1399.01c* during nitrogen starving conditions in both PJ512 and PJ513 within 40 min and a plateau was

reached after 80 – 100 min. No differences in the expression were seen between the two strains.

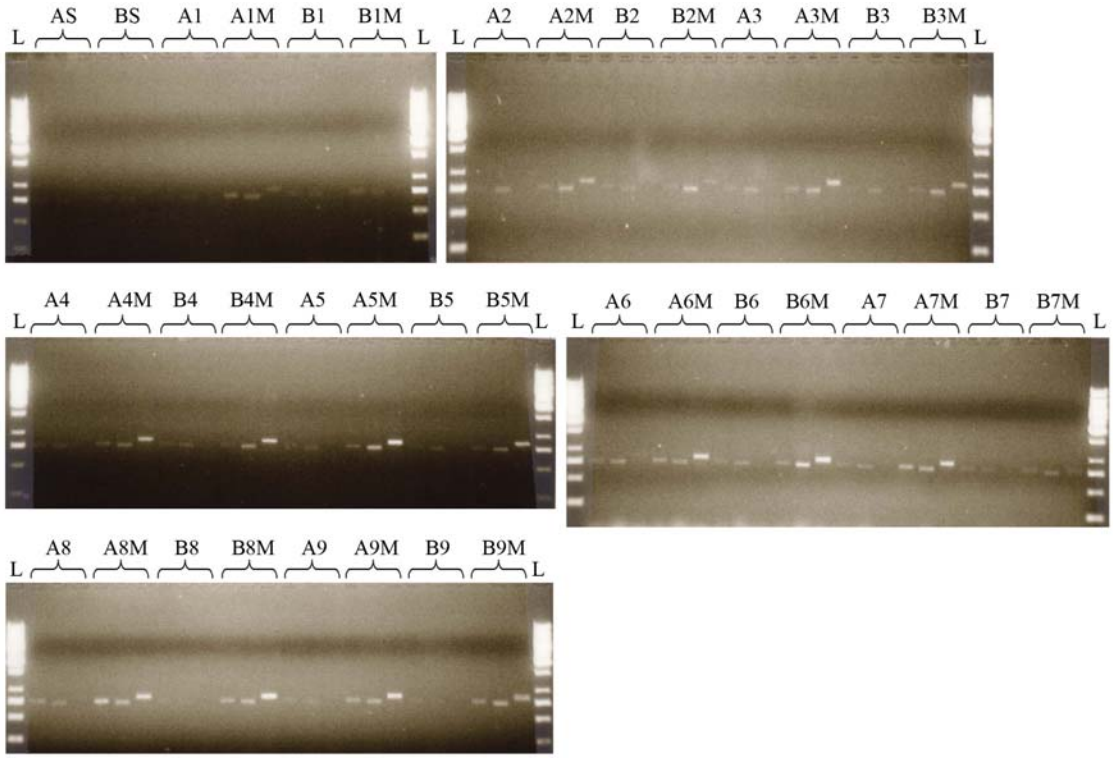

Figure 9. Gel photos showing PCR products from the PCR after RT-PCR in experiment 2 on PJ512 and PJ513 to quantify the expression of the genes *ste11*, *fur4* and *SPAC1399.01c*. The same primers were used as described in Figure 8. A means PJ512 and B means PJ513. M means cells grown in EMM –ura $-NH_4Cl$ and no M means cells grown in EMM –ura. S means that the sample was taken at the start, before the wash, 1 means sample taken at t=0 (nitrogen starvation start), 2 at t=20 min, 3 at t=40 min, 4 at t=60 min, 5 at t=80 min, 6 at t=100 min, 7 at t=120 min, 8 at t=140 min and 9 at t=160 min. The three lanes next to each other correspond to PCR using three different set of primers: Ste11F/R (*ste11*), Chr1clusterMF/R (*fur4*) and Chr1clusterSF/R (*SPAC1399.01c*). An upregulation of all the three genes is seen in both PJ512 and PJ513 during nitrogen starvation.

3.7 Microscopy

The insertion of *lacO* repeats at the two gene clusters in both the h^{90} and h^{+} strain background was successful and was verified by PCR in all transformed strains. The successful transformations were also verified by seeing a bright GFP signal in the fluorescence microscope. Another important thing was that the autofluorescence (background) from the *S. pombe* cells was very low and this is not always the case. Figure 10 shows a clear GFP signal in PJ512. The YFP ring however, was not visible in the microscope used.

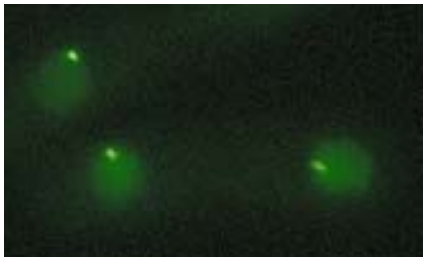

Figure 10. In this photo the Chr1cluster is visualised in PJ512. The GFP signal is very bright. The background signal is probably nonbound LacR-GFP inside the cell nucleus.

4 Discussion

4.1 Introduction

This work aimed to visualise the localisation of two clusters containing nitrogen starvation genes, Subtel1 and Chr1cluster, in *S. pombe* and detect possible localisation changes between them during vegetative growth and nitrogen starvation. The strategy to reach the goal was to visualise the clusters using the *lacO*/LacR-GFP recognition system and to visualise the nuclear membrane using Pom152-YFP. The distance between the GFP signal and the nuclear membrane was then supposed to be measured. To verify that the genes in the cluster really were upregulated during nitrogen starvation, RNA was to be analysed by RT-PCR. Unfortunately, the goal was not reached but many important results were obtained.

4.2 Conclusions

This was the first successful attempt to introduce the *lacO*/lacR-GFP recognition system in this lab. Strong GFP-signals were seen in all the transformed strains using fluorescence microscope (Figure 10). This work also shows through the RT-PCR experiment that genes in Chr1cluster cluster really were upregulated due to nitrogen starvation within 40 min after the induction in haploid cells and that a plateau was reached after 80 -100 min using the quantification method described. The received knowledge gained and together with the proposed improvements discussed below, conditions for studying localisation of clustered nitrogen starvation genes in *S. pombe* were created.

4.3 Strains and transformants

The original plan was to cross strains with Pom152-YFP (PJ370) and LacR-GFP and then transform these strains first with *ura4C* and then with the complete *ura4*$^+$ gene together with the *lacO* repeats. Crossing strains is time consuming and to save time, transformations were made on strains only containing LacR-GFP and hence the strains PJ488, PJ489, PJ487 and PJ484 containing *ura4C* and LacR-GFP and PJ522, PJ525, PJ521 and PJ519 containing *ura4C*, *lacO* repeats and LacR-GFP were created. These strains could later obtain Pom152-YFP by crossing with PJ370. Possible alternatives to using Pom152-YFP that was considered was to stain the DNA (in the nucleus) with Hoechst staining to be able to see the nucleus, alternatively to get a high background of nonbound LacR-GFP in the cell nucleus. Only one of the strains, PJ519, was crossed with PJ370 to produce PJ508. When strains with both LacR-GFP and Pom152-YFP were obtained these were transformed and proceeded with. Although one of the final experiments (nitrogen starvation, experiment 1) was performed on PJ508.

Sometimes it was hard to find correct transformants. Prior to colony PCR it was confirmed that the candidates grew on hygromycin plates (electroporation) or PMG –ura plates (chemical transformation). But the growth on hygromycin plates only confirmed that the hygromycin gene was transformed into the cells, not that it was inserted at the right locus in the genome. The same argument was true for the chemical transformation; growth on PMG –ura plates did not confirm insertion at the right locus. This led to a lot of false positives that had to be excluded by colony PCR. Sometimes it was enough to screen ten transformants, sometimes as many as 36 transformants from one transformation had to be screened.

4.4 Microscopy

The GFP signals in the transformed cells were seen very clearly in the fluorescence microscope and there were hardly any autofluorescence from the cells. It was not possible to detect the YFP signal due to weak signal, bleaching of the signal and difficulties in optimising the settings for the microscope and the software. Due to overlapping emission wavelengths for GFP and YFP it was impossible to detect the different signals separately. One possibility to try to solve these problems is to use a confocal microscope, where the excitation wavelength could be set. Another possibility is to use CFP (cyan fluorescent protein) instead of YFP. The emission wavelengths of CFP do not overlap with GFP and the CFP signal is stronger than the YFP signal.

The changing of filters from the YFP filter to the GFP filter was done manually and when doing so the microscope and the objective (the cells) were distorted. When the YFP and GFP pictures were superimposed the distortion was clearly observed because the GFP signals (seen in both the GFP and YFP channels) only partly overlapped. The object moved about 0.2 μm which corresponds to about 10% of the diameter of the nucleus and that was unacceptable. To solve this problem a microscope that changes filters mechanically could be used and this will not disturb the object, or a confocal microscopy could be used.

Only the GFP signal was analysed due to time restraints and inadequate qualities of the microscope. I hoped that the LacR-GFP in the nucleus not bound to *lacO*, would give a sufficient background to discern the limits of the nucleus (nuclear envelope) and hence get a reference point. This was performed in PJ512 and PJ513. Several pictures (z-stacks) were taken of each object and the stacks were then used to build a 3D image. In this image it was possible to see the GFP signal, but not as a dot, more as a sausage. When looking at the 3D image it was impossible to discern if the GFP signal that looked as nonbound in the 2D picture really were nonbound GFP since it was not spread in the whole nucleus. There are good chances to succeed in solving these problems as soon as the settings are optimised for the newly bought software. Different settings for taking pictures, measuring in 3D and in digital deconvolution will help in gaining informative pictures. As an alternative a confocal microscope can be used instead.

To sum up these suggestions, it is probably possible to analyse the created strains with LacR-GFP and Pom152-YFP. It might be possible to use the background of nonbound LacR-GFP to visualise the extent of the nucleus instead of the weak YFP signal. To get another reference point than the YFP signal or the LacR-GFP background signal, Pom152 can be labelled by CFP instead or the DNA in the nucleus can be stained by Hoechst staining. A microscope that changes filters mechanically has to be used. The best way to analyse these cells in the future is probably by using a confocal microscope.

4.5 Future

In the introduction it was described that the Subtel1 region is affected by the histone deacetylase Clr3, an enzyme believed to be involved in heterochromatin regulation [24] and therefore it would be of interest to investigate possible differences in location of Subtel1 in strains with a deleted *clr3* gene. But first it has to be investigated if and when genes in Subtel1 cluster are upregulated during nitrogen starvation in haploid h^+ cells.

As mentioned in the introduction, methylation of lysine 9 on histone H3 is associated with heterochromatin and methylation of lysine 4 on histone H3 is associated with euchromatin. A study made by Cam and co-workers [36] have mapped the methylations of lysine 9 and lysine

4 on histone H3 throughout the entire genome of *S. pombe*. They also mapped where the protein Swi6 is associated with the chromatin (heterochromatin). They found that H3 lys9 and Swi6 are concentrated at the centromeres, telomeres and at two small regions associated with genes expressed during meiosis. In the future it would be interesting to study the localisation of the meiotic genes that are associated with H3lys9 and Swi6 in the study of Cam and co-workers.

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7 Litterature

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Appendix 1: Meiosis induced genes in Chr1cluster and Subtel1

Chr1cluster

Biological name	Systematic name	Expression pattern/biological process
	SPAC1399.04C	induced by nitrogen starvation or pheromone signalling, transient
	SPAC1399.05C	induced by nitrogen starvation or pheromone signalling, transient
	SPAC1399.02	induced by nitrogen starvation or pheromone signalling, transient
	SPAC1399.01C	induced by nitrogen starvation or pheromone signalling, transient
fur4	SPAC1399.03	induced by nitrogen starvation or pheromone signalling, transient
	SPAC1002.18	induced by nitrogen starvation or pheromone signalling, transient
	SPAC1002.19	spore formation

Subtel1

Biological name	Systematic name	Expression pattern/biological process
	SPAC11D3.09	induced by nitrogen starvation or pheromone signalling, transient
	SPAC11D3.08C	induced by nitrogen starvation or pheromone signalling, transient
	SPAC11D3.16C	induced by nitrogen starvation or pheromone signalling, transient
	SPAC11D3.17	induced by nitrogen starvation or pheromone signalling, transient
	SPAC977.13C	induced by nitrogen starvation or pheromone signalling, transient
	SPAC11D3.03C	induced by nitrogen starvation or pheromone signalling, transient
	SPAC1F8.04C	induced by nitrogen starvation or pheromone signalling, continuous
dak2	SPAC977.16C	induced by nitrogen starvation or pheromone signalling, delayed
ght3	SPAC1F8.01	premeiotic S phase and recombination
	SPAC13G6.08	meiotic division
chs1	SPAC13G6.12C	meiotic division
	SPAC11D3.10	meiotic division
	SPAC24B11.05	meiotic division
	SPAC212.02	meiotic division
	SPAC212.04C	meiotic division
	SPAC212.01C	meiotic division
	SPAC1348.01	meiotic division
	SPAC977.06	meiotic division
isp3/meu4	SPAC1F8.05	meiotic division
	SPAC212.05C	meiotic division
	SPAC5H10.11	spore formation
	SPAC977.01	spore formation
	SPAC11D3.13	spore formation
	SPAC5H10.02C	spore formation
	SPAC11D3.01C	spore formation

Appendix 2: Media

Rich media

YEA 1 l (l)

5 g Bacto™ Yeast Extract (Becton, Dickinson and Company)
2 g Bacto™ Casamino Acids (Becton, Dickinson and Company)
0.1 g Adenine (SIGMA)
0.25 g L-Leucine (SIGMA)
0.1 g Uracil (SIGMA)

The bottle was filled up with sterilised deionised water to 850 ml and then sterilised by autoclaving. After sterilising 30 g D(+)Glucose-Monohydrat (MERCK) (150 ml from a stock solution of 20% glucose , sterilised separately by autoclaving) was added.

Solid media was made by adding 20 g Bacto™ Agar (Becton, Dickinson and Company) before autoclaving.

Selective rich media

YE

YE was prepared as YEA but by excluding adenine.

YEA G418

YEA G418 was prepared as YEA and 200 mg/l G418 disulfate (Duchefa) was added after autoclaving. The bottle with media was allowed to cool for a while before adding G418.

YEA hygromycin

YEA hygromycin was prepared as YEA and 100 mg/l or 200 mg/l hygromycin B (AppliChem) was added depending on the desired concentration to the media after autoclaving. The bottle with media was allowed to cool for a while before adding hygromycin.

Minimal media

PMG total 1 l (l)

3 g KH phthalate, SigmaUltra (SIGMA)
2.76 g $Na_2HPO_4 \cdot 2H_2O$ (MERCK)
3.74 g L-Glutamic acid (BDH)
0.25 g L-Leucine (SIGMA)
0.1 g Adenine (SIGMA)
0.1 g Uracil (SIGMA)
0.1 g L-Histidine (SIGMA)
0.1 g L-Lysine (SIGMA)
0.1 ml Minerals stock (10000x) (see below)
1 ml Vitamins stock (1000x) (see below)
20 ml Salts stocks (50x) (see below)

The bottle was filled up with sterilised deionised water to 900 ml and then sterilised by autoclaving. After sterilising 20 g D(+)Glucose-Monohydrat (MERCK) (100 ml from a stock solution of 20% glucose, sterilised separately by autoclaving) was added.

Solid media was made by adding 20 g (2% w/v) Bacto™ Agar (Becton, Dickinson and Company) before autoclaving but after adjusting pH to about 5.6.

Minerals stock (10000x)
8.1 µM H_3BO_3
2.37 µM $MnSO_4$
1.39 µM $ZnSO_4 \cdot 7H_2O$
0.74 µM $FeCl_3 \cdot 6H_2O$
0.25 µM $MoO_4 \cdot 2H_2O$
0.6 µM KI
0.16 µM $CuSO_4 \cdot 5H_2O$
4.76 µM Citric acid
The contents were dissolved in deionised water and then filter sterilised.

Vitamins stock (1000x)
4.2 mM Pantothenic acid
81.2 mM Nicotinic acid
55.5 mM Inositol
40.8 µM Biotin
The contents were dissolved in deionised water and sterilised by autoclaving.

Salts stock (50x)
5.2 mM $MgCl_2 \cdot 6H_2O$
0.1 mM $CaCl_2 \cdot 2H_2O$
13.4 mM KCl
0.28 mM Na_2SO_4
The contents were dissolved in deionised water and sterilised by autoclaving.

EMM
EMM was prepared as PMG but instead of L-Glutamic acid, 5 g of NH_4Cl (MERCK) was added. The glucose added to this media was sterile filtered instead of autoclaved to reduce background for microscopy studies.

Selective minimal media

EMM –ura
EMM –ura was prepared as EMM but uracil was excluded.

Nitrogen starvation media
EMM –ura –NH_4Cl was prepared as EMM but uracil and NH_4Cl were excluded.

PMG -leu, -ade, -ura, - his, -lys
PMG -leu, -ade, -ura, - his, -lys were prepared as PMG but by excluding corresponding amino acid/nitrogenbase.

Appendix 3: PCR programs

JTAG

1. 94°C for 2 min
2. 94°C for 15 s
3. 47°C for 30 s
4. 68°C for 4 min
5. repeat from step 2, 9 times (10 times total)
6. 94°C for 15 s
7. 47°C for 30 s
8. 68°C for 4 min, increase this time with 15 s each cycle
9. repeat from step 6, 19 times (20 times total)
10. 4°C hold

JPCR

1. 94°C for 2 min
2. 94°C for 30 s
3. 55°C for 15 s
4. 70°C for 1 min 30 s
5. repeat from step 2, 29 times (30 times total)
6. 4°C hold

JPCR21

7. 94°C for 2 min
8. 94°C for 30 s
9. 55°C for 15 s
10. 70°C for 1 min 30 s
11. repeat from step 2, 20 times (21 times total)
12. 4°C hold

JPCR23

13. 94°C for 2 min
14. 94°C for 30 s
15. 55°C for 15 s
16. 70°C for 1 min 30 s
17. repeat from step 2, 22 times (23 times total)
18. 4°C hold

Appendix 4: Primers

Contstruction of *ura4C*

ura4 C-term:1: 5' – CGC GGA TCC GAA TTG TTG GCT TTG ATG GAA G – 3'

ura4 C-term:2: 5' – CGC GGA TAA TTA GTC GCT ACA TAA AAT TTT ACC – 3'

Restriction site for BamHI is underlined.
Product: 891 bp

Construction of plasmid pFA6a-*ura4C*-hphMX6

Subtel1F: 5' – TGA AAA GAC ATT CTG TTT GGT TTG ATT TAT TGC ATA CGT TAC TAG CGC AGT CTT CAA CAA TTT TTC GTA TAG ATT AAA ATT TAA GTG ACT TTT TCT ATA CCG GAT CCC CGG GTT AAT TAA – 3'

Subtel1R: 5' – ACA AAC AAA GCA ATG TGA AAA CAA GAA GAC AAA AGG AAT TTT ACT TGT CGT CGA CTC TTT TGG GAA TTT TTT AAT AAC AAT CAA ACC AAC AAA AGA AAG AAG TGA ATT CGA GCT CGT TTA AAC – 3'

Chr1clusterF: 5' – CAG TAT TAG AAA AGG TAC CGC GAA ATT CCA AAA TCT GAA AGG AAA TAC AAT TCC CCA AGC TAT TAA GGA GAA GAA AAA AAA GAT GAA TAT ATG GAT GTT GCG GAT CCC CGG GTT AAT TAA – 3'

Chr1clusterR: 5' – AAG TTG TCC AAT CAT ATT ATT TTT TAG AAC TAA TAT TCA ATC ATT GCA AGC TAT TTC CAG ATA TCC GGA TGC ATC CTC AAC CGA TCA GCC GGG TTA TAT AGA ATT CGA GCT CGT TTA AAC – 3'

The part of the primers that is complementary to the pFA6a-hphMX6 vector is underlined. The remaining part has homology to genomic *S. pombe* DNA [35].

Verification of transformants

CtrlSubtel1F: 5' – CTC AAA GTG AGC AAC GTA C – 3'

CtrlSubtel1B: 5' – CTT ATA ATC TGA TAG TAT GTT C – 3'

CtrlChr1clusterF: 5' – GCA GCG ATA TTA AGT TTT GG – 3'

CtrlChr1clusterR: 5' – CAT CCA CTA CTT AAA TTT GTC – 3'

Ura4F: 5' – GAG GGG ATG AAA ATT CCC A – 3'

Ura4R: 5' – TTC GAC AAC AGG ATT ACG AC – 3'

Quantitative PCR after RT-PCR

Primers for *ste11* gene
Ste11F: 5’ – CTC GGA TTA CGT TGT ACT G – 3’

Ste11R: 5’ – GTA TGG CAA TAA GGC GTA G – 3’

Primers for *fur4* gene
Chr1clusterMF: 5’ – GTA ACT TCG CTA ATC GGT G – 3’

Chr1clusterMR: 5’ – CTG TCC TTG TCG ATG GAG – 3’

Primers for *SPAC1399.01c* gene
Chr1clusterSF: 5’ – GAT TCC AAC ACG CTT TAG C – 3’

Chr1clusterSR: 5’ – CAT CAT GTT TAC AAT GTA CAG – 3’

Optimal $MgCl_2$ concentration for all of the primers was 2 mM for Expand High fidelity PCR system (Roche).

Appendix 5: RNA concentrations

Experiment 1

Experiment performed on PJ508 cells.
M means cells grown in EMM –ura –NH4Cl and no M means cells grown in EMM –ura. 1 means sample taken at t=0 (nitrogen starvation start), 2 at t=20 min, 3 at t=40 min, 4 at t=60 min, 5 at t=80 min, 6 at t=100 min and 7 at t=120 min.

Sample	A_{260} nm	Dilution factor	Concentraion ng/µl	Mean value ng/µl
1	0,377	50	754	761
1	0,386	50	772	
1	0,378	50	756	
1M	0,361	50	722	736
1M	0,371	50	742	
1M	0,372	50	744	
2	0,367	50	734	735
2	0,370	50	740	
2	0,366	50	732	
2M	0,364	50	728	738
2M	0,379	50	758	
2M	0,364	50	728	
3	0,509	50	1018	1033
3	0,518	50	1036	
3	0,522	50	1044	
3M	0,324	50	648	631
3M	0,312	50	624	
3M	0,311	50	622	
4	0,169	50	338	357
4	0,170	50	340	
4	0,197	50	394	
4M	0,300	50	600	589
4M	0,293	50	586	
4M	0,290	50	580	
5	0,674	50	1348	1395
5	0,713	50	1426	
5	0,705	50	1410	
5M	0,321	50	642	653
5M	0,331	50	662	
5M	0,328	50	656	
6	0,510	50	1020	1020
6M	0,511	50	1022	1024
6M	0,513	50	1026	

Sample	A_{260} nm	Dilution factor	Concentraion ng/µl	Mean value ng/µl
7	0,543	50	1086	1089
7	0,557	50	1114	
7	0,533	50	1066	
7M	0,463	50	926	903
7M	0,450	50	900	
7M	0,442	50	884	

Experiment 2

Experiment performed on A=PJ512 and B=PJ513.
M means cells grown in EMM –ura –NH4Cl and no M means cells grown in EMM –ura. S means sample taken at start, before wash, 1 means sample taken at t=0 (nitrogen starvation start), 2 at t=20 min, 3 at t=40 min, 4 at t=60 min, 5 at t=80 min, 6 at t=100 min, 7 at t=120 min, 8 at t=140 min, 9 at t=160 min.

Sample	A_{260} nm	Dilution factor	Concentraion ng/µl	Mean value ng/µl
AS	0,067	25	67	65
AS	0,059	25	59	
AS	0,068	25	68	
BS	0,071	25	71	66
BS	0,067	25	67	
BS	0,059	25	59	
A1	0,075	25	75	76
A1	0,076	25	76	
A1M	0,043	25	43	45
A1M	0,046	25	46	
B1	0,074	25	74	74
B1	0,073	25	73	
B1M	0,050	25	50	60
B1M	0,070	25	70	
A2	0,075	25	75	84
A2	0,085	25	85	
A2	0,093	25	93	
A2M	0,054	25	54	58
A2M	0,068	25	68	
A2M	0,051	25	51	
B2	0,086	25	86	89
B2	0,087	25	87	
B2	0,094	25	94	
B2M	0,065	25	65	66
B2M	0,067	25	67	
B2M	0,066	25	66	
A3	0,096	25	96	87
A3	0,071	25	71	
A3	0,095	25	95	
A3M	0,054	25	54	64
A3M	0,066	25	66	
A3M	0,072	25	72	
B3	0,061	25	61	69
B3	0,078	25	78	
B3	0,069	25	69	
B3M	0,052	25	52	50
B3M	0,054	25	54	
B3M	0,044	25	44	

Sample	A_{260} nm	Dilution factor	Concentraion ng/µl	Mean value ng/µl
A4	0,079	25	79	86
A4	0,093	25	93	
A4	0,087	25	87	
A4M	0,043	25	43	45
A4M	0,044	25	44	
A4M	0,048	25	48	

Experiment 2 continued

Sample	A_{260} nm	Dilution factor	Concentraion ng/µl	Mean value ng/µl
B4	0,068	25	68	65
B4	0,052	25	52	
B4	0,075	25	75	
B4M	0,058	25	58	61
B4M	0,061	25	61	
B4M	0,065	25	65	
A5	0,039	25	39	35
A5	0,035	25	35	
A5	0,031	25	31	
A5M	0,051	25	51	65
A5M	0,075	25	75	
A5M	0,070	25	70	
B5	0,068	25	68	67
B5	0,065	25	65	
B5	0,068	25	68	
B5M	0,043	25	43	48
B5M	0,043	25	43	
B5M	0,058	25	58	
A6	0,091	25	91	93
A6	0,095	25	95	
A6	0,092	25	92	
A6M	0,030	25	30	32
A6M	0,037	25	37	
A6M	0,029	25	29	
B6	0,048	25	48	52
B6	0,059	25	59	
B6	0,048	25	48	
B6M	0,049	25	49	51
B6M	0,052	25	52	
B6M	0,051	25	51	
A7	0,094	25	94	108
A7	0,116	25	116	
A7	0,115	25	115	
A7M	0,053	25	53	55
A7M	0,059	25	59	
A7M	0,054	25	54	
B7	0,064	25	64	77
B7	0,061	25	61	
B7	0,106	25	106	
B7M	0,044	25	44	43
B7M	0,044	25	44	
B7M	0,042	25	42	

Sample	A_{260} nm	Dilution factor	Concentraion ng/µl	Mean value ng/µl
A8	0,081	25	81	77
A8	0,062	25	62	
A8	0,088	25	88	
A8M	0,083	25	83	85
A8M	0,087	25	87	
A8M	0,085	25	85	
B8	0,080	25	80	85
B8	0,087	25	87	
B8	0,087	25	87	
B8M	0,090	25	90	96
B8M	0,102	25	102	
B8M	0,095	25	95	
A9	0,109	25	109	111
A9	0,108	25	108	
A9	0,116	25	116	
A9M	0,072	25	72	77
A9M	0,076	25	76	
A9M	0,083	25	83	
B9	0,041	25	41	42
B9	0,042	25	42	
B9	0,044	25	44	
B9M	0,077	25	77	78
B9M	0,074	25	74	
B9M	0,084	25	84	