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Identification and characterization of genes and mutants for an *N*-terminal acetyltransferase from yeast

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A gene from *Saccharomyces cerevisiae* has been mapped, cloned, sequenced and shown to encode a catalytic subunit of an N-terminal acetyltransferase. Regions of this gene, *NAT1*, and the chloramphenicol acetyltransferase genes of bacteria have limited but significant homology. A *nat1* null mutant is viable but exhibits a variety of phenotypes, including reduced acetyltransferase activity, derepression of a silent mating type locus (*HML*) and failure to enter G_0. All these phenotypes are identical to those of a previously characterized mutant, *ard1*. *NAT1* and *ARD1* are distinct genes that encode proteins with no obvious similarity. Concomitant overexpression of both *NAT1* and *ARD1* in yeast causes a 20-fold increase in acetyltransferase activity *in vitro*, whereas overexpression of either *NAT1* or *ARD1* alone does not raise activity over basal levels. A functional iso-1-cytochrome *c* protein, which is N-terminally acetylated in a *NAT1* strain, is not acetylated in an isogenic *nat1* mutant. At least 20 other yeast proteins, including histone H2B, are not N-terminally acetylated in either *nat1* or *ard1* mutants. These results suggest that NAT1 and ARD1 proteins function together to catalyze the N-terminal acetylation of a subset of yeast proteins.

Key words: Saccharomyces cerevisiae/*N*-terminal acetyltransferase/gene/mutant

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Table I. Acetyltransferase activity in NAT1 and *nat1-1* strains from two tetrads

Tetrad	Activity (c.p.m.)[a]		*NAT1*	*TRP1*
	25°C	37°C		
2a	3850	1100	+	−
2b	3200	350	−	+
2c	3450	500	−	+
2d	3700	1100	+	−
3a	3300	950	+	−
3b	3450	1100	+	−
3c	3800	350	−	+
3d	3000	400	−	+

[a]Acetyltransferase assays were performed as described in Materials and methods. Assays at 37°C were with extracts that had been pre-heated at 45°C for 8 min, while the assays at 25°C were with unheated extracts.

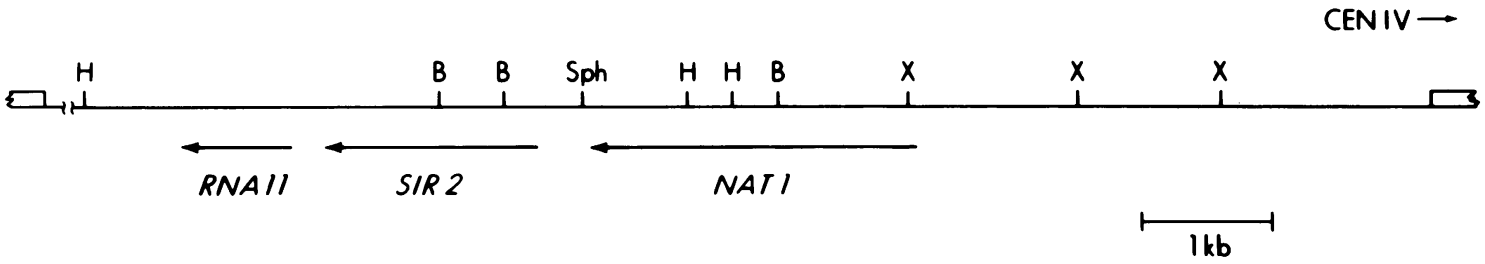

Fig. 1. Restriction map of pJM100. pJM100 contains ~15 kb of genomic DNA, including three genes: *NAT1*, *SIR2* and *RNA11*. The map order of these three genes with respect to the centromere (*CENIV*) is indicated. The map order was determined by tetrad analysis from crosses of appropriately marked haploid strains (see Materials and methods). Restriction sites are abbreviated as follows: *Bam*HI (B), *Xho*I (X), *Hin*dIII (H), *Sph*I (Sph).

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NAT1: 148  RKKYWEAFLGYRANWTSLAVAQDV  171
           **    ***             *   *
 CAT:  18  RKEHFEAFQSVAQCTYNQTVQLDI  41

NAT1: 681  GEKLIETS-TPMED-FATEFYNNYS--MQVREDERDY  713
           **   *   *   *   *       *           * *
 CAT:  80  GELVIWDSVHPCYTVFHEQTETFSSLWSEYHDDFRQF  116
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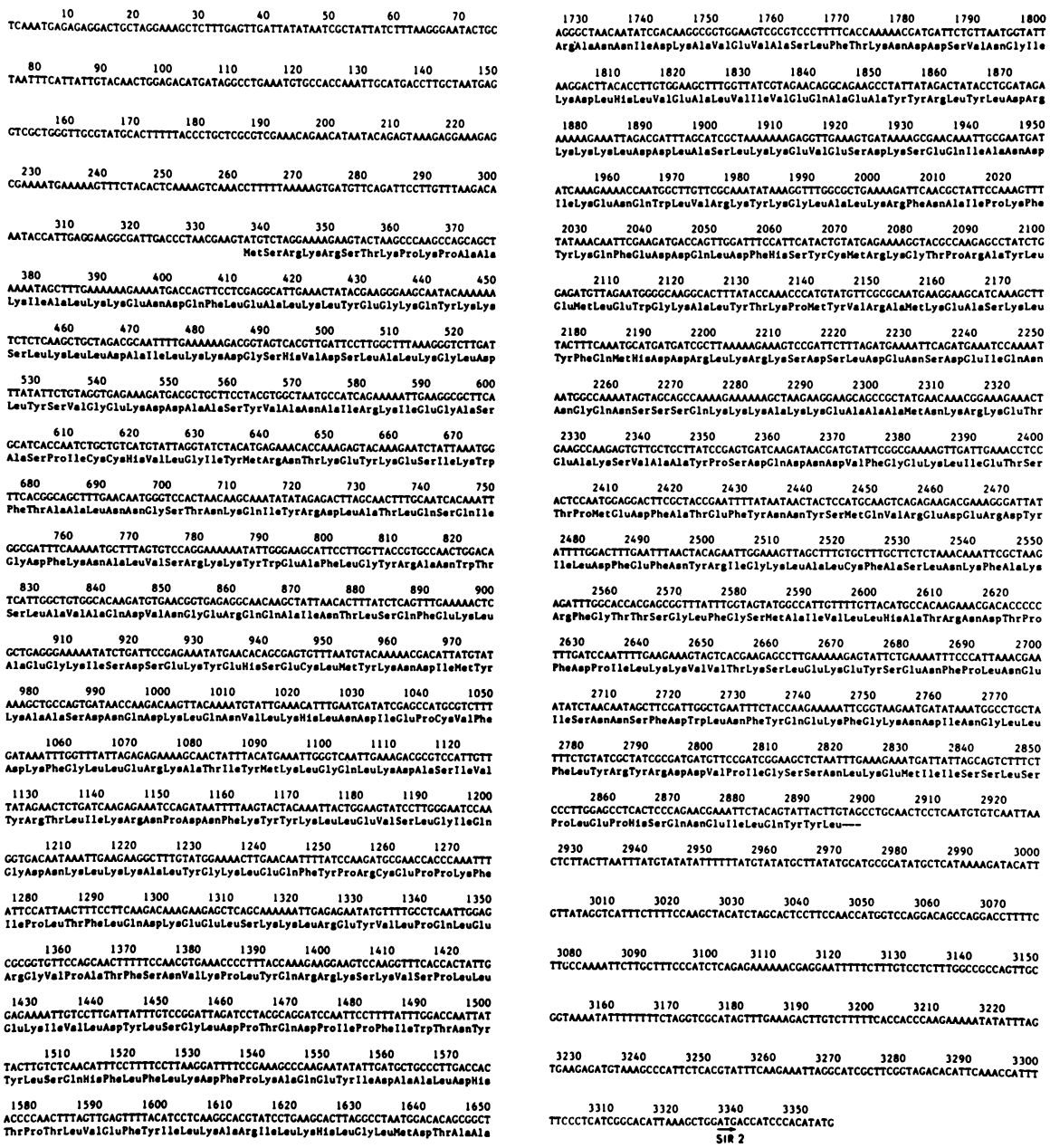

Fig. 2. DNA sequence of *NAT1*. The sequence of *NAT1* from nucleotide 1 to nucleotide 2975 was determined by standard methods (see Materials and methods). The sequence from nucleotides 2976 to 3354 is from Shore *et al.* (1984). The beginning of the *SIR2* ORF is indicated by an arrow. There is complete agreement in the overlap region from nucleotides 2710 to 2975, sequenced by both our group and Shore *et al.* (1984). The putative protein sequence of *NAT1* is indicated.

Table II. Effect of *nat1* mutation on survival to heat shock and mating efficiency

Strain	Relevant genotype	% Survival after heat shock[a]	Relative mating efficiency[b]
W303-1a	*NAT1*	91	1.0
AMR1	*nat1*	18	6.6×10^{-3}

[a]Strains were grown in YPD at 30°C for 4 days and then subjected to a 5-min heat shock at 54°C as described in Materials and methods.
[b]Strains were grown to mid-log phase in YPD at 30°C. Dilutions were made and aliquots were plated onto either YPD plates (to determine the number of viable cells) or onto SD plates spread with a thin lawn of the MATα strain 217 (to determine mating ability). The mating efficiency is the number of cells that mated per total number of cells; it was arbitrarily defined as 1.0 for W303-1a.

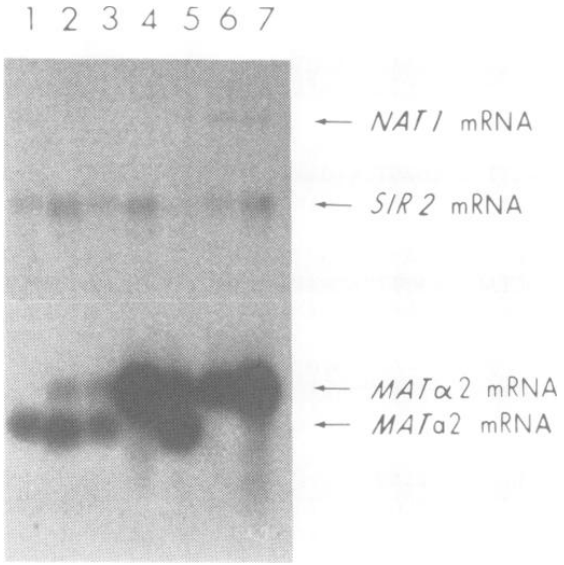

Fig. 3. Derepression of *HML* in *nat1* and *ard1* single mutants and in *nat1 ard1* double mutant strains. Total RNA was probed for α2 and a2 transcripts, using ^{32}P-labeled anti-sense RNA. **Lanes 1** and **2**, *MAT*a *NAT1* (W303-1a) and *MAT*a *nat1* (AMR1), respectively, — an isogenic set; **lanes 3** and **4**, *MAT*a *nat1 ard1* and *MATα nat1 ard1* double mutants, respectively; **lane 5**, *MAT*a *sir1 sir3* strain (RS19); **lanes 6** and **7**, *MATα ARD1* (9-3A) and *MATα ard1* (9-4D), respectively, — an isogenic set. Note the darker α2 transcript band in the *MATα ard1* strain (**lane 7**) and the *MATα nat1 ard1* double mutant (**lane 4**). This is due to partial derepression of *HML* in addition to normal transcription from the *MAT* locus. This effect is also seen in *MATα nat1* strain (data not shown). The same filter was probed with a ^{32}P-labeled anti-sense SIR2 probe as a control. A short region of this riboprobe also contained anti-sense *NAT1* RNA (see Materials and methods), thus also allowing visualization of the *NAT1* transcript.

Table III. Acetyltransferase activities of *nat1* and *ard1* single and double mutants

Strain	Relevant genotype	Acetyltransferase activity (c.p.m.)[a]	
		No pre-heating	8 min at 45°C
RS304-1a	*NAT1 ARD1*	12000	6050
RS304-1b	*NAT1 ard1*	10400	2400
RS304-1c	*nat1 ARD1*	8250	2500
RS304-1d	*nat1 ard1*	9200	2600

[a]Assays were for 20 min at 25°C as described in Materials and methods.

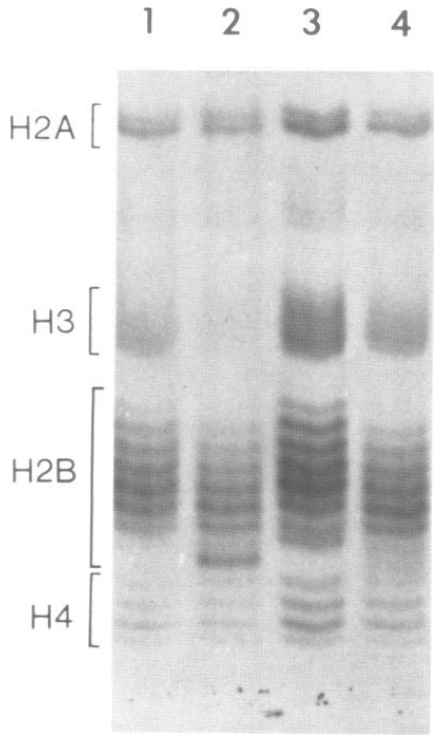

Fig. 4. ^{3}H-Labeled histones from *nat1* and *ard1* single mutants and their isogenic parents. Yeast histones were labeled *in vivo* with [^{3}H]acetate, isolated and separated on a 12% Triton−acid−urea gel, which was then processed for fluorography. **Lane 1**, strain W303-1a (*NAT1*); **lane 2**, AMR1 (*nat1*); **lane 3**, 9-3A (*ARD1*); **lane 4**, 9-4D (*ard1*). The histone H2B bands from the *nat1* mutant (**lane 2**) and the *ard1* mutant (**lane 4**) are shifted down by one 'step', a movement consistent with the loss of the N-terminal acetyl group. The very dark band low in the H2B group of the *nat1* mutant (**lane 2**) is not seen in the *ard1* mutant (**lane 4**). This difference in the pattern is probably due to the fact that the strains are not isogenic.

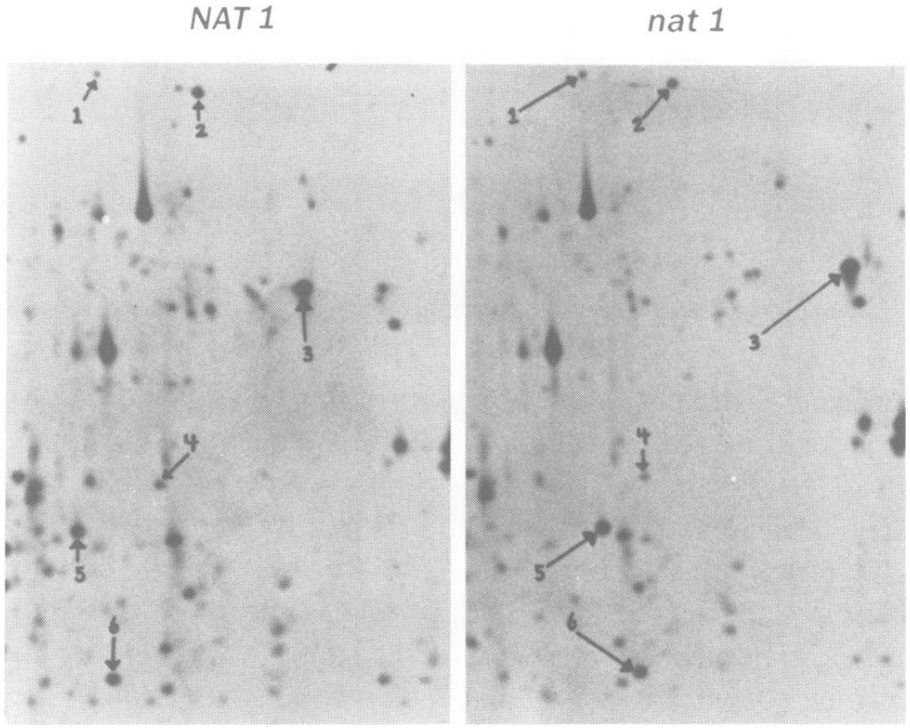

Fig. 5. Two-dimensional gels of [^{35}S]methionine-labeled proteins from *NAT1* and *nat1* strains. *NAT1* (W303-1a) and *nat1* (AMR1) strains were briefly labeled with [^{35}S]methionine, total protein was isolated and separated on two-dimensional gels, which were then processed for fluorography. The first dimension is an isoelectric focusing gel; the basic side is to the right. The second dimension is a standard denaturing gel with the direction of migration from top to bottom. The regions shown contain proteins of ~30–80 kd, with pIs ranging from ~4 to 8. Identical regions from the two gels are shown. Six prominent protein spots are indicated that shift toward the basic side in the *nat1* strain. The shifts are consistent with lack of acetylation of the N termini of these proteins in the *nat1* strain.

Table IV. Overproduction of acetyltransferase activity *in vitro*

Plasmids	Overproduced protein(s)	Specific activity (c.p.m./μg protein)[a]
pVT100-U + pVT103-L	–	1550
pVT100-U + pJM124	NAT1	1500
M33p5 + pVT103-L	ARD1	1150
M33p5 + pJM124	NAT1 + ARD1	33 700

[a]Extracts were prepared as described in Materials and methods, but were not subjected to pre-heating prior to assaying. Assays were at 25°C for 20 min. Extracts were diluted 1:10 and 1 μl was used per assay. With undiluted extracts, the NAT1/ARD1 overproducing extract gave 8–10 times more activity than the other extracts.

Materials and methods

Yeast strains, media and strain manipulations

Yeast were grown at 30°C in either YPD medium or in SD medium plus appropriate supplements (Sherman *et al.*, 1986). Cells were transformed by standard spheroplasting techniques (Sherman *et al.*, 1986). Diploid construction, sporulation, tetrad dissection and analysis were done by standard methods (Sherman *et al.*, 1986). The following strains were used: YS7p/4d: *MATα ade2 ura3 leu2 trp1 his3*; MCSY314: *MAT*a *ade ura1-1 lys2-1 thr4 met4-1 can1-100 nat1-1*; W303-1a: *MAT*a *ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100*; AMR1: Isogenic to W303-1a plus *nat1-5* :: *LEU2*; J24: *MAT*a *ade1 ade2 ura1 his7 lys2 tyr1 gal1-1 rna11*^{ts}; 9-3A: *MATα ura3 leu2 his3*; 9-4D: isogenic to 9-3A plus *ard1* :: *URA3* (Whiteway *et al.*, 1987); RS19: *MAT*a *sir1-1 sir3* :: *LEU2 ade6 arg4 leu2 trp1 RME1*; DF1: *MAT*a *ade2 ura3-52 trp1-1 lys2 his3 his7 leu2-3,112 NAT1* :: *LEU2*; DF2: *MATα ade2-101 ura leu2-3,112 tyr1 his rna11*^{ts} *lys2*; 216: *MAT*a *his1*; 217: *MATα his1*; B-7470: *MATα CYC1-793 cyc7-67 lys5-10 ura3-52*; B-7658: isogenic to B-7470 plus *nat1-3* :: *URA3*.

Null mutations and plasmids

The *nat1-3* :: *URA3* null mutation was constructed by deleting a *Hin*dIII fragment from the *NAT1* coding sequence (see Figure 1) and inserting a 1.1 kb *Hin*dIII fragment containing the *URA3* gene. The mutation was placed in the genome by gene transplacement (Rothstein, 1983). The construction of the *nat1-5* :: *LEU2* null mutation is described in the Results. M33p5 (a gift from M.Whiteway) contains the *ARD1* gene cloned behind the constitutive ADH promoter in a 2 μm based *URA3* vector (Vernet *et al.*, 1987). pJM124 contains a functional *NAT1* gene (coding for the first 11 amino acids of phage T7 gene 10 protein plus four amino acids from linker sequences, followed by amino acids 7−853 of *NAT1*) cloned behind the ADH promoter in a 2 μm based *LEU2-d* plasmid (Vernet *et al.*, 1987).

Extracts and acetyltransferase assays

In the original screen for a histone acetyltransferase mutant, cells were grown in 20 ml of YPD to a density of 1×10^7 cells/ml, pelleted, resuspended in 1 ml assay buffer (75 mM Tris pH 8.2, 150 mM NaCl, 0.1 mM EDTA, 2 mM β-mercaptoethanol), and vortexed with an equal volume of glass beads in 30s pulses alternated with 1 min on ice for a total vortexing time of 4 min. Disrupted cells were centrifuged at 12 000 *g* for 10 min and pellets were resuspended in 0.225 ml assay buffer. Aliquots containing 0.5 mg total protein (Lowry *et al.*, 1951) were used in assays (Travis *et al.*, 1984). After the original screening, NAT assays including those shown in Tables I, III and IV, were done in the following manner. Strains were grown in 50 ml of YPD to 2×10^8 cells/ml or in 100 ml of minimal medium to 1×10^8 cells/ml. Cells were pelleted, washed once with water, and made into spheroplasts using the protocol of Celniker and Campbell (1982). All manipulations from this point on were done at 4°C or on ice. Spheroplasts were transferred to Eppendorf tubes, washed with 1 ml of Buffer A (Celniker and Campbell, 1982) and pelleted. Nuclei were released by homogenization (30 strokes of a micro-pestle) in 0.1 ml Buffer A and pelleted in a microfuge. Nuclei were lysed by addition of 0.2 ml of NAT YLB [20 mM Tris pH 7.4, 10% glycerol, 1 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF]. After brief vortexing, the crude nuclear lysates were centrifuged for 10 min in a cold microfuge, and the supernatants used for assays. One μl of crude extract (containing between 3 and 7 μg protein/μl) was used per 50 μl assay. Assay conditions were those of Travis *et al.* (1984) except that extracts were placed at 45°C for 8 min just prior to assaying. Assays were at 25°C for 20 min. Reactions were stopped by the addition of 1.5 ml of cold 20% TCA. Precipitated proteins were collected on glass fiber filters (Schleicher and Schuell), washed twice with 5% TCA, once with methanol, dried and counted.

Mapping of NAT1, SIR2 and RNA11

The linkage of *NAT1* and *TRP1* was established by crossing strain MCSY314 to YS7p/4d. The resulting tetrads were scored for the *nat1-1* mutation (by the *in vitro* acetyltransferase assay) and for tryptophan auxotrophy by replica plating. The linkage of *NAT1* and *RNA11* was established by crossing a *nat1-1* mutant strain to J24. The resulting haploid progeny were analyzed for both acetyltransferase activity *in vitro* and for temperature sensitivity.

The map order of the three genes *NAT1*, *SIR2* and *RNA11* with respect to the centromere of chromosome IV was determined by inserting the *LEU2* gene into the *Xho*I site furthest upstream from *NAT1* (see Figure 1), and replacing wild-type DNA with this marked DNA by gene transplacement (Rothstein, 1983). This haploid strain, DF1 (constructed by D.Fossaceca), was then mated to an appropriately marked strain, DF2, creating a diploid which was heterozygous for the three markers: *rna11*^{ts}, *trp1* and *LEU2* (marking the region upstream of *NAT1*). This diploid was sporulated and the resulting tetrads analyzed for crossovers between *LEU2* (*NAT1*) and *rna11*^{ts}. Analysis of 157 tetrads resulted in five crossovers between the marked region upstream of *NAT1* and *rna11*^{ts}. In all five cases, *LEU2* (*NAT1*) remained parental with respect to *TRP1*, while *RNA11* became tetratype with respect to both *TRP1* and *LEU2* (*NAT1*), indicating that the map order is *RNA11-SIR2-NAT1*-CENIV-*TRP1*, as shown in Figure 1.

Sequencing of NAT1 and homology search

Overlapping restriction fragments from the *NAT1* coding region were subcloned into either M13mp10 or M13mp11 (Messing and Vieira, 1982) permitting sequencing of both strands. Sequence analysis of the subclones was carried out by the dideoxy method of Sanger *et al.* (1977).

The *NAT1* sequence was compared to every sequence in the Protein Identification Resource databases (PIR, National Biomedical Research Foundation, 3900 Reservoir Road, N.W., Washington, DC 20007). These included PSQ (release 14.0, comprising 4721 sequences with a total of 1 118 494 residues) and New (release 31.0, comprising 1697 sequences with a total of 393 114 residues). Sequence alignments were peformed using the BESTFIT program of the GCG sequence analysis package (University of Wisconsin Genetics Computer Group, U. of W. Biotechnology Center, 1710 University Ave., University of Wisconsin, Madison, WI, 53705). A variety of gap (G) and gap length (L) penalties were examined: G = 2.0, 5.0, and 7.5; L = 0.0, 0.05, 0.1, and 1.0. Sequence alignments between individual protein sequences, as opposed to searches of the PIR databases, were performed both with the entire sequence and with 50- or 100-residue segments of one of the sequences. Profile analysis was carried out by the method of Gribskov *et al.* (1987), using logarithmic weighting applied uniformly to each sequence used to generate the profile. Searches of the PIR databases were repeated three times with different values of gap and gap length weights: 3.0, 0.1 (G,L); 5.0, 0.05; and 10.0, 0.0.

Phenotype tests

α-Factor sensitivity was determined by adding α-factor (Sigma) to a final concentration of 5 μg/ml to logarithmically growing yeast cultures in pH 5.0 YPD medium at a density of $1-2 \times 10^7$ cells/ml. Three to four hours later, cells were examined microscopically for formation of mating structures. Quantitative matings were done by plating serial dilutions of logarithmically growing cells onto SD plates containing a lawn of either 216 or 217 mating type tester strains to determine diploid formation. Serial dilutions were also plated onto YPD plates to determine total cell number. Heat shock assays were performed by diluting stationary cultures (4–5 days in YPD at 30°C) to 1×10^8 cells/ml in water, subjecting them to 54°C for 5 min, cooling them quickly on ice and plating serial dilutions on YPD plates to determine viability.

CYC1 protein isolation and sequencing

Iso-1-cytochrome *c* was isolated and sequenced as previously described (Moerschell *et al.*, 1988).

Northern blots and RNA probes

RNA was isolated according to standard procedures (Nasmyth, 1983) and Northern blots were performed as previously described (Brill and Sternglanz, 1988). The α2/a2 probe, pGEM3B-α2, was constructed by inserting a 2.3 kb *Xba*I–*Hin*dIII fragment from *MATα* into the *Xba*I–*Hin*dIII cut pGEM3Blue riboprobe vector (Promega, Madison, WI). This plasmid was linearized with *Hin*dIII and anti-sense RNA was generated using T7 RNA polymerase. This riboprobe also anneals to a2 transcripts. The *SIR2* probe, pGEM3B-SIR2, was constructed by inserting a 2.0 kb *Bgl*II–*Hin*dIII fragment from the *SIR2* gene into *Bam*HI–*Hin*dIII cut pGEM3Blue. This plasmid was linearized with *Hin*dIII and anti-sense RNA was generated with T7 RNA polymerase. The *SIR2* fragment used also contains a small part of the *NAT1* gene, so riboprobes generated by T7 RNA polymerase are able to anneal weakly to *NAT1* transcripts as well as to *SIR2* transcripts.

^{3}H-Labeling of yeast histones

Cells were grown in 100 ml YPD to 2 × 10^7 cells/ml and made into spheroplasts by standard techniques (Sherman *et al.*, 1986). Spheroplasts were resuspended in 2 ml labeling buffer, incubated with 2 mCi [^{3}H]acetate (Amersham, 2–5 Ci/mmol) according to Nelson (1982), in the presence of 100 μg/ml cycloheximide (Sigma). Pelleted spheroplasts were resuspended in ice-cold NIB (Alonso and Nelson, 1986) with a Triton X-100 concentration of 0.5%. Spheroplasts were incubated for 15–30 min on ice and pelleted (5500 *g*) for 8 min at 4°C. NIB incubation followed by pelleting was repeated twice. The crude nuclear pellet was resuspended in cold 100 mM Tris pH 6.8, 0.4 M NaCl, 1 mM PMSF and incubated for 10 min on ice. Nuclei were pelleted (5500 *g*, 8 min, 4°C) and the salt wash was repeated with a 5 min incubation on ice. Pelleted nuclei were then resuspended in 1.35 ml cold water containing 1 mM PMSF. One tenth volume (0.15 ml) of 4 N H_2SO_4 was added while swirling and the histones were extracted on ice overnight. Slurries were centrifuged 10 min at 12 000 *g* at 4°C. Supernatants were transferred to 15 ml Falcon tubes, filled with cold acetone/HCl (acetone:5 M HCl, 99:1), mixed, and put at −20°C overnight. Histones were pelleted at 10 000 *g* for 10 min. Pellets were lyophilized and resuspended in loading buffer for Triton–acid–urea gels.

Triton–acid–urea gels

12% Acrylamide (30:0.2) gels (13 cm long, 14 cm wide, 1.5 mm thick) were made containing 8 M urea (IBI), 0.37% Triton X-100, 0.9 N acetic acid, 0.125% ammonium persulfate, and 0.125% TEMED (Alfageme *et al.*, 1974; Zweidler, 1978). After polymerization, gels were pre-electrophoresed for at least 6 h at 150 V, until amperage was constant. Wells were loaded with 1 M cysteamine (Sigma), 8 M urea, 0.9 N acetic acid and the gels were electrophoresed for another hour at 150 V. Wells were cleaned and fresh buffer was placed in both upper and lower chambers. Histones in loading buffer (8 M urea, 10% glycerol, 0.9 N acetic acid, 5% BME, 0.25% methyl green) were boiled for 5 min, cooled and loaded on gels. Histones were electrophoresed at 115–125 V for 19–21 h, until the blue component of the dye was at the bottom of the gel. Gels were stained with Coomassie Blue R (Sigma) and prepared for fluorography (Bonner and Laskey, 1974; Laskey and Mills, 1975).

[^{35}S]Methionine labeling of proteins

Strains were grown overnight in supplemented SD medium lacking methionine and then diluted to a density of $\sim 5 \times 10^6$ cells/ml. When the culture reached $\sim 2 \times 10^7$ cells/ml, 1 ml of cells was labeled with 0.1 mCi of [^{35}S]methionine (Dupont) for 15 min at 30°C. Labeled cells were transferred to an Eppendorf tube and washed twice with ice-cold water. The cells were resuspended in 0.1 ml water and an equal volume of 0.45 μm glass beads was added. Cells were vortexed for 10 s and 0.1 ml of 0.6% SDS, 2% BME, 100 mM Tris pH 8.0 (maintained at 100°C) was added. The mixture was vortexed for 1 s and 0.1 ml was removed (avoiding glass beads) and added to an Eppendorf tube on ice containing 10 μl RNase/DNase solution (1 mg/ml DNase, 0.5 mg/ml RNase, 500 mM Tris pH 7.0, 50 mM $MgCl_2$). Protein samples were incubated for 1 min on ice and then frozen at −70°C.