THE *ILV1* GENE IN *SACCHAROMYCES CEREVISIAE*: 5' and 3' END MAPPING OF TRANSCRIPTS AND THEIR REGULATION

by

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Keywords: Threonine deaminase, nuclease S1, primer extension, 5' heterogeneity, promoter, general amino acid control

The *ILV1* gene of *Saccharomyces cerevisiae* encodes threonine deaminase, the first enzyme in the biosynthesis of isoleucine. The 5' and 3' termini of the *ILV1* mRNA have been mapped by primer extension using AMV reverse transcriptase and S1 nuclease. A considerable heterogeneity was found at the 5' end, which displays twenty different termini spanning a region of 78 nucleotides (from position -39 to -116 upstream of the ATG translation initiation codon). S1 nuclease mapping reveal one 3' end 143 to 147 nucleotides downstream to the last codon in the 1,728 bp long *ILV1* coding region. The two most prevalent transcripts comprise about half of the *ILV1* mRNA and initiate at -39 and -40 at the sequence CAAG, not previously found to be a cap site in genes coding for amino acid biosynthesis enzymes. All the twenty 5' termini are located within a 93 nucleotide open reading frame with its ATG at the symmetry point of a large imperfect tandem inverted repeat. The steady state level of the *ILV1* mRNA is derepressed in response to amino acid starvation (general amino acid control). The general control consensus sequence TGACT is found at positions -22, -132 and -307, the latter two being located upstream to the mRNA initiation sites. The relative abundance of mRNA molecules with different 5' termini in cells derepressed to various extent was very similar. Three TATA like sequences and a CAAT box can be recognized upstream to the transcribed region. No intervening sequences were found to be present in the *ILV1* transcription unit indicating that the entire 1,728 bp open reading frame is translated.

1. INTRODUCTION

In *Saccharomyces cerevisiae* five enzymes catalyse the biosynthesis of isoleucine and valine (35, 41). The first enzyme in the pathway for isoleucine is threonine deaminase (threonine

dehydratase, 4.2.1.16.) which converts threonine to α -ketobutyrate. The other four enzymes are shared by the biosynthetic pathway for valine. The immediate precursor for valine, α -ketoisovaleric acid, serves as the starting point

Abbreviations: AMV = avian myeloblastosis virus; bp = base pairs; kb = kilobase pairs; ORF = open translational reading frame.

for the synthesis of leucine (57).

Threonine deaminase consists of four subunits with a molecular weight of approximately 50,000 (2) and may be complexed in the mitochondria with other isoleucine-valine enzymes (54). The structural gene for the enzyme, *ILV1*, is nuclear and located on chromosome V. The amount of threonine deaminase in the cell is regulated. Compared to the level found in cells grown in minimal medium, repression of its synthesis is observed by the addition of isoleucine, valine and leucine (resembling multivalent repression in *E. coli*) (13, 23, 40). Also, its synthesis is derepressed in response to amino acid starvation (general amino acid control) (34).

This paper continues our analyses of the *ILV1* gene of *S. cerevisiae*. We have previously reported on the cloning of the gene by functional complementation in yeast. The presence of the gene on the cloned DNA fragment was verified genetically and its approximate position on the restriction endonuclease map was determined by Northern blot analysis using subfragments as probes (49). Subsequent DNA sequencing revealed a 1,728 base pair long open translational reading frame (37). The size of the deduced polypeptide is larger than the experimentally determined molecular weight of the enzyme monomer. This indicates that a peptide with a molecular weight of about 15,000 is cleaved off in connection with the intracellular processing of the enzyme.

Here we present a detailed transcriptional analysis of the *ILV1* gene. The positions in the DNA sequence that correspond to the 5' end and the 3' end of the messenger have been determined and suggest that the full 1,728 base pair open reading frame is translated. Furthermore, sequences flanking the 5' end of the mRNA with possible importance for transcription initiation by RNA polymerase II and for regulation by general amino acid control are discussed.

2. MATERIALS AND METHODS

2.1. Strains and media

The *S. cerevisiae* wild type strains were S288C (*MAT α SUC2 gal2 CUP1*) and X2180-1A (*MAT α SUC2 mal mel gal2 CUP1*). Strain

X2180-1A was kindly provided by P. Niederberger, Zürich, Switzerland. The *E. coli* strains were HB101 (*recA hsdS*), BJ5183 (*recBC sbcB endoI hsd*) (61), RRI Δ M15 (*leu pro thi strA hsd r' m' lacZ Δ M15 F'*lacI o* Δ M15 pro'*) (53) and JM101 (44).

The media used for growth of yeast (50) and *E. coli* (33) have been described. In repression and derepression studies, amino acids were added as described in section 2.4.

2.2. Chemicals and enzymes

All chemicals were analytical grade. Restriction endonucleases, T4 DNA ligase, calf alkaline phosphatase, Klenow large fragment DNA polymerase I, T4 polynucleotide kinase, exonuclease VII and S1 nuclease were from Boehringer (Mannheim, Germany), Bethesda Research Laboratories (Bethesda, Maryland, USA) and Amersham International (Amersham, England). Avian myeloblastosis virus (AMV) reverse transcriptase was from Life Sciences (St. Petersburg, Florida, USA). All enzymes were used as recommended by the companies, unless otherwise noted. The pentadecamer sequencing primer was from New England Biolabs (Beverly, Massachusetts, USA) and radionucleotides [α - 32 P]dATP (> 600 Ci/mmol) and [γ - 32 P]ATP (> 7000 Ci/mmol) were from New England Nuclear (Massachusetts, USA).

2.3. DNA preparation

Plasmid DNA from *E. coli* was prepared in small scale by the alkaline extraction procedure of BIRNBOIM and DOLY (10). Large scale plasmid preparations and the replicative form of the filamentous phage vector M13mp8, were made by a scaled up version of the alkaline extraction procedure followed by CsCl equilibrium centrifugation. Preparation of phage particles and single-stranded phage DNA were performed as described (45, 55, 56). Isolation of DNA restriction fragments from agarose gels by electroelution onto a dialysis membrane was according to YANG et al. (66). The fragments were further purified by phenol extraction and gel filtration on Sephadex G-50 (Pharmacia, Uppsala, Sweden).

2.4. RNA preparation

RNA was isolated from the wild type strains after growth at 30 °C in liquid minimal medium or minimal medium supplemented with the amino acids indicated in the text at 5 mM concentrations. The cells were harvested at a density of $1\text{--}2 \times 10^7$ cells $\cdot$ ml⁻¹. For derepression of amino acid biosynthetic enzymes 5-methyl-DL-tryptophan at a final concentration of 0.5 mM was added to the cells growing in minimal medium ($2\text{--}5 \times 10^6$ cells $\cdot$ ml⁻¹), whereafter the cells were incubated for 7 hours before harvesting. Thiaioleucine, an analogue of isoleucine and threonine, was used in the same manner as 5-methyl tryptophan, but at a concentration of 5 mM. For the preparation of total RNA the method of BROACH et al. (12) was used with modifications: Cells from 100 ml of culture were pelleted, washed once with 10 ml of H₂O and resuspended in 2 ml of lysis buffer (25 mM-Tris-HCl pH 7.4, 25 mM-NaCl, 5 mM-MgCl₂, 250 μ g $\cdot$ ml⁻¹ heparin). Lysis was accomplished by the addition of 2 volumes of glass beads (0.45 mm³) and 2 ml of phenol:chloroform:isoamyl alcohol (24:24:1 v/v) followed by 2 minutes of vortexing. Ten ml each of the lysis buffer and the organic solvent mixture were then added and extraction was carried out for 10 minutes. Extraction was repeated until the interphase was clear and the RNA was ethanol precipitated. When RNA was made from 1 liter of culture the volumes were multiplied by a factor of 5. Enrichment for poly(A) RNA was performed by a single step of chromatography on poly(U) Sepharose (Pharmacia, Uppsala, Sweden).

2.5. Transformation and subcloning

E. coli cells were transformed according to COHEN et al. (17). Selection of recombinant pUR250 plasmid derivatives or recombinant M13 phages were performed as described (53, 55, 56). The following plasmid/phage constructions with fragments from the *ILV1* region (cf. Figure 1) were used as sources for hybridization probes: pC517 is pBR322 with a 2.8 kb HindIII/SalI fragment inserted between the HindIII and SalI sites, pC550 is pUR250 with a 1.55 kb EcoRI fragment derived from pC517 inserted into the EcoRI site, pC518 is pBR322 with a 3 kb

HindIII fragment inserted into the HindIII site, mp8-551 is M13mp8 with a 441 bp HindIII/PstI fragment derived from pC518 inserted between the HindIII and PstI sites.

2.6. Nucleotide sequencing and gel electrophoresis

Nucleotide sequencing of single-stranded M13 recombinant DNA molecules was performed by the dideoxy chain termination technique according to SANGER et al. (55, 56). Gel electrophoresis of sequencing reactions, reverse transcripts, S1 nuclease and exonuclease VII resistant DNA fragments were run on 6%, 8% or 10% polyacrylamide gels containing urea. The gels were fixed, dried and autoradiographed as described by RASMUSSEN et al. (51).

2.7. Preparation of hybridization probes

Restriction fragments were 3' end-labelled with [α -³²P]dATP and Klenow large fragment DNA polymerase I. Labelling of the 5' end was by treatment with alkaline phosphatase and incubation with [γ -³²P]ATP and T4 polynucleotide kinase. End-labelling procedures as well as strand separation were carried out as described by MAXAM and GILBERT (42). The preparation of highly radioactive probes from mp8-551 was a modification of the procedure of MCKNIGHT (43): One pmole of the sequencing primer was added to 0.25 pmole of the single-stranded mp8-551 in 10 μ l of 10 mM-Tris-HCl pH 7.4, 10 mM-MgCl₂, 50 mM-NaCl in a sealed glass capillary. The capillary was boiled for 5 minutes, incubated in a water bath at 65 °C for 15 minutes and the water bath allowed to cool to room temperature. Chain elongation of the annealed template/primer mixture was carried out with 2.5 units of Klenow large fragment of DNA polymerase I in a total volume of 20 μ l with 20 μ Ci of [α -³²P]dATP (corresponding to 50 pmol) and in the presence of 1 nmol of each of the deoxynucleotides dGTP, dCTP and dTTP. After incubation at room temperature for 30 minutes the reaction was chased for 5 minutes with 1 μ l of 0.5 mM-dATP. From this sample two different probes were obtained: Half of the double-stranded DNA was digested with 5 units PstI in

Figure 1. Structural and transcriptional map of the *ILV1* region in *S. cerevisiae* (S288C). The DNA sequence from position -777 to +1989 has been determined by KIELLAND-BRANDT et al. (37). The DNA fragments subcloned and prepared for use in the present study are indicated. ----- means prepared single-stranded probe. ~~~~~ designate 37 nucleotides of M13 vector sequences in the single-stranded probes.

40 μ l to isolate a 478 base single-stranded DNA bounded by the primer and the PstI site of the insert (P-1) and the other half with 5 units RsaI in 40 μ l to isolate a 333 base single-stranded DNA bounded by the primer and the RsaI site of the insert (R-1, cf. Figure 1). After digestion for 1 hour at 37 $^{\circ}$ C, the DNA's were heated at 90 $^{\circ}$ C for 3 minutes in formamide-dye buffer and electrophoresed on a 1 mm thick denaturing 6% polyacrylamide gel. After detection of the radioactive bands on X-ray film, gel slices containing the single-stranded DNA were excised and eluted. To the eluate were added 5 μ g of tRNA from *E. coli* and the nucleic acids were ethanol precipitated. The precipitate was dissolved in 200 μ l of 10 mM Tris-HCl pH 8, 0.5 mM-EDTA, phenol extracted and further purified by gel filtration. Specific activities of about 5×10^8 dpm $\cdot \mu$ g $^{-1}$ were obtained.

2.8. Reverse transcription, nuclease S1 and exonuclease VII mapping

For reverse transcription (4) 10^5 dpm of single-

stranded probe was mixed with either total RNA or poly(A) enriched RNA in amounts as indicated in the text, phenolized and ethanol precipitated. The nucleic acids were dissolved in 25 μ l of hybridization buffer (40 mM-Pipes-NaOH pH 6.4, 1 mM-EDTA, 0.4 M-NaCl, 80% (v/v) formamide) sealed in a glass capillary, heated at 75 $^{\circ}$ C for 10 minutes and then hybridized at 42 $^{\circ}$ C overnight. Three volumes of water were added and the material precipitated with ethanol. Formamide was removed by two additional ethanol precipitations. The hybrids were then dissolved in 50 μ l of 100 mM-Tris-HCl pH 8.3, 10 mM-MgCl $_2$, 140 mM-KCl, 20 mM- β -mercaptoethanol and 1 mM each of the four non-radioactive deoxynucleotides and reacted with 54 units of AMV reverse transcriptase for 1 hour at 43 $^{\circ}$ C. The reaction mixture was made 20 mM in EDTA, divided into two fractions and ethanol precipitated. One half was subjected to digestion with nuclease S1 (see below). The other half was dissolved in 10 μ l 0.1 M-NaOH, 5 mM-EDTA and 10 μ l formamide-dye buffer were added and aliquots were electrophoresed on 6%, 8% or 10%

polyacrylamide sequencing gels.

Nuclease S1 mapping was modified from BERK and SHARP (9). Approximately 10^5 dpm of either single-stranded or double-stranded probe were mixed with the amount of RNA indicated in the text, phenolized, precipitated, taken up in 25 μ l of hybridization buffer, heated at 75 °C for 10 minutes and hybridized overnight. In the case of double-stranded probes the hybridization temperatures were 45 °C, 49 °C and 52 °C, whereas 40 °C or 42 °C was used with single-stranded probes. The DNA:RNA mixture was diluted into 275 μ l of cold S1 buffer (50 mM-Na-acetate pH 4.6, 4.5 mM-ZnSO₄, 0.28 M-NaCl, 20 μ g $\cdot$ ml⁻¹ of denatured, phenolized salmon sperm DNA) and incubated at 30 °C for 40 minutes with 100 units of S1 nuclease, or at 37 °C for 30 minutes with 500 units of S1. Digestion was stopped by addition of 2 μ l of 0.5 M-EDTA and 10 μ g of carrier E. coli tRNA followed by precipitation with ethanol. In the case of the double-stranded probes (2.8 kb) the S1 resistant DNA fragments were analysed on a 0.7% alkaline agarose gel as described by McDONNELL et al. (39), while the S1 resistant fragments resulting from experiments with single-stranded probes were analysed on 6%, 8% or 10% sequencing gels.

Digestion with exonuclease VII of DNA:RNA hybrids was done as described for nuclease S1, but employing exonuclease VII buffer (30 mM-KCl, 10 mM-EDTA, 10 mM-Tris-HCl (pH 7.9)) and 3 units of exonuclease VII at 37 °C for 30 minutes.

3. RESULTS

3.1. Direction of transcription of the *ILV1* gene

In two recent reports we have described the cloning and sequencing of the *S. cerevisiae* *ILV1* gene (37, 49). We found one open reading frame of 1,728 bp and that the *ILV1* transcript of about 2,000 nucleotides covered this region. A summary of these data is shown in the upper section of Figure 1.

The study of the transcription of *ILV1* was initiated before the sequence was determined and therefore the direction of transcription of

Figure 2. Direction of transcription of the *ILV1* coding region. Top: Schematic description of the experiment. Twenty μ g of poly(A) enriched yeast RNA was hybridized with 5×10^4 dpm of 3' and 5' end-labelled DNA fragments at 49 °C and digested with 500 units of S1 nuclease at 37 °C for 30 minutes. The radioactive, S1 resistant DNA fragments were analysed by electrophoresis on a 0.7% alkaline agarose gel followed by autoradiography. Bottom: Autoradiograph of the 0.7% denaturing agarose gel. Lane 1: λ DNA digested with EcoRI and HindIII and 3' end labelled as size markers. Lane 2 and 3: The untreated 3' and 5' end-labelled HindIII/SalI fragments, respectively. Lane 4: S1 nuclease digestion of the 5' end-labelled DNA:RNA duplexes. Lane 5: S1 nuclease digestion of 3' end-labelled DNA:RNA duplexes.

the gene was determined employing the S1 nuclease protection mapping technique of BERK and SHARP (9). The experiment is depicted diagrammatically in Figure 2. The 2.8 kb HindIII/SalI fragment from the *ILV1* region cloned in pC517 (cf. Figure 1) was isolated and labelled either at the 3' ends with [α - 32 P]dATP or at the 5' ends with [γ - 32 P]ATP. The HindIII site is internal to the 2,000 nucleotide mRNA whereas the SalI site is outside its right-hand endpoints and the *ILV1* mRNA is the only transcript from this DNA (49). Each of the end-labelled fragments were then mixed with 20 μ g poly(A) enriched RNA isolated from strain S288C grown in minimal medium, ethanol precipitated and resuspended in 80% formamide buffer. After heating at 75 °C for 10 minutes to denature the DNA, hybridization was performed at 45 °C, 49 °C and 52 °C, respectively, in order to find the optimal conditions for RNA:DNA hybrids (15). After digestion with S1 nuclease the DNA:RNA mixtures were subjected to electrophoresis in an 0.7% alkaline agarose gel and protected, radioactive single-stranded DNA visualized by autoradiography. The optimal temperature for DNA:RNA hybrid formation was found to be 49 °C, and the result using this temperature is shown in Figure 2. It is seen that the fragment 5' end-labelled in the HindIII site was not protected by any RNA species (lane 4), whereas the fragment 3' end-labelled in the same HindIII site was protected by an 1.6 kb RNA species (lane 5). The fragment 5' end-labelled in the SalI site will be protected by the same 1.6 kb RNA piece, but will not be radioactive after digestion with S1. From this result we can conclude that the transcription of the *ILV1* mRNA is initiated 5' to the HindIII site and that the direction of transcription is towards the SalI site. This agrees with the DNA sequence (37). The experiment also locates the 3' end of the mRNA to be about 1,600 bases downstream of the HindIII site. The experiment does not exclude the existence of several closely spaced ends. The results also indicate that no intervening sequences occur within this 1.6 kb part of *ILV1*.

3.2. *ILV1* transcription initiation sites

Since the experiment described above posi-

tioned the 3' end of the mRNA about 1,600 nucleotides downstream of the HindIII site, the region of transcription initiation is presumed to occur about 400 bases upstream to the HindIII site, i.e. within the 441 bp HindIII/PstI fragment (cf. Figure 1). In order to locate the start of transcription the following experiments were made. The mRNA identical strand of the 441 bp HindIII/PstI fragment was cloned in M13mp8, giving mp8-551, and the regular pentadecamer sequencing primer was used to synthesize the complementary strand. The primer was extended about 300 nucleotides using [α - 32 P]dATP whereafter synthesis was continued with non-radioactive dATP. Digestion of this double-stranded DNA with either PstI or RsaI was followed by preparative electrophoresis on denaturing polyacrylamide gels. This resulted in two single-stranded probes designated P-1 and R-1. Probe P-1 consists of the 441 base HindIII/PstI fragment (cf. Figure 1) with the addition of 37 extra nucleotides which consists of the 22 bases separating the pentadecamer sequencing primer from the HindIII site and the primer itself. Probe R-1 consists of the 296 base HindIII/RsaI fragment (cf. Figure 1) with the same extra 37 nucleotides. Probe R-1 was used to prime RNA-dependent DNA synthesis employing AMV reverse transcriptase: Twenty μ g of poly(A) enriched yeast RNA or 80 μ g of total yeast RNA was hybridized with 10⁵ dpm of R-1 at 42 °C in 80% formamide overnight. After extension by reverse transcriptase half of the reaction mixture was precipitated with ethanol and resuspended in NaOH-formamide-dye buffer. The other half was first subjected to S1 nuclease digestion and then prepared for electrophoresis in the same way. Aliquots of the denatured samples were analysed on a 6% sequencing gel. As illustrated in Figure 3, primer extension required the presence of a yeast RNA template; with *E. coli* tRNA no alteration of the primer, R-1, can be seen (lane 3). The shorter molecules seen in lane 3 result from isotope decay and are also seen in lane 4 (primer alone). When yeast poly(A) enriched RNA (lane 1) or total yeast RNA (lane 2) is used as the source of template the same gel pattern is obtained which reproducibly reveals at least 12 different products, corresponding to

Figure 3. 5' termini of *ILVI* mRNA analysed by primer extension and nuclease S1 mapping. Lanes 1, 2 and 3 show primer extension reactions using the R-1 fragment (333 nucleotide *RsaI*/*HindIII* single-stranded fragment, cf. Figure 1). The primer was used to probe for the following template molecules. Lane 1: 20 μ g of poly(A) enriched yeast RNA, lane 2: 80 μ g of total yeast RNA, lane 3: 10 μ g of *E. coli* tRNA. Lane 4 is the untreated R-1 probe. Lanes 5, 6 and 7 show digestion with 100 units of nuclease S1 of the primer extension mixtures shown in lanes 1, 2 and 3, respectively. Lanes 8 and 9 show digestion with 100 units of S1 of hybridizations between probe P-1 (478 nucleotide *PstI*/*HindIII* single-stranded fragment, cf. Figure 1) and either 80 μ g of total yeast RNA (lane 8) or 10 μ g of *E. coli* tRNA (lane 9). Lane 10 presents the untreated P-1 probe. The size markers are ϕ X174 RF DNA digested with *HinfI* and 3' end labelled. Electrophoresis was on a 6% polyacrylamide/urea sequencing gel.

Figure 4. A: Mapping of the 5' termini of the *ILV1* mRNA by reverse transcription employing a 5' end-labelled 194 nucleotide DdeI/FokI single-stranded primer (D-1, cf. Figure 1). The primer was hybridized with the following RNAs. Lane 1: 20 μ g of yeast poly(A) enriched RNA. Lanes 2, 3, 4 and 5: 3 μ g, 10 μ g, 50 μ g and 160 μ g of total yeast RNA, respectively. Lane 6: 10 μ g of *E. coli* tRNA. The sequence of the relevant part of the *ILV1* region is shown on the right. In the sequence the nucleotides in bold face indicate the positions of the 5' ends. B: Relative abundance of the 5' termini of the *ILV1* mRNA during various growth conditions. Reverse transcription employing the D-1 primer as in A after hybridization with the following RNAs: Lane 1: 20 μ g of poly(A) enriched yeast RNA extracted from cells grown in minimal medium. Lanes 2-7 are with 50 μ g total yeast RNA from cells grown in minimal medium with the following additions. Lane 2: None, lane 3: Isoleucine (5 mM), lane 4: Isoleucine (5 mM) and valine (5 mM), lane 5: Isoleucine, valine and leucine, lane 6: 5-methyl tryptophan (0.5 mM), lane 7: Thiaisoleucine (5 mM). In both A and B the sizes of the DNA fragments were determined by dideoxy chain termination sequence reactions (not shown) and electrophoresis was on a 6% polyacrylamide/urea sequencing gel.

the addition of between 75 and 150 nucleotides (the bands from 410 to 485 bases). The most prominent band (about 410 bases) reflects an extension of about 75 nucleotides and constitutes roughly 50% of the total extension products. The faint bands seen between the primer and the 410 bp extension band are regarded as decay products. To verify that the extended DNA strands are faithful copies of the *ILV1* mRNA the extension mixtures were digested with nuclease S1. The results (lanes 5 and 6) show that all the extended DNA strands as well as the unextended primer of the RNA:DNA hybrids are S1 nuclease resistant. It should be noted that treatment with S1 nuclease, as expected, has reduced the unextended primer as well as the extended primer by the 37 nucleotides of M13mp8 sequence. In conclusion, the pattern of bands obtained in lanes 1, 2, 5 and 6 indicates, in addition to a major mRNA species, a considerable heterogeneity at the 5' end of this transcript spanning a region of about 75 nucleotides.

We also used an S1 nuclease assay (9) to identify the 5' ends employing the P-1 probe: Eighty µg of total yeast RNA was hybridized with 10⁵ dpm of P-1 at 42 °C in 80% formamide buffer overnight, the DNA:RNA mixture was digested with nuclease S1 and after ethanol precipitation the protected DNA's were analysed on a 6% sequencing gel (Figure 3, lane 8). The band pattern is identical to the pattern obtained by S1 nuclease digestion of the R-1 primer extension products: A very prominent band with the size of 370 nucleotides as well as at least 10 bands spanning the region from 370 to 445 bases. The fact that the same protected DNA strands are obtained by S1 digestion of R-1 primer extension products and S1 treatment of the RNA:DNA hybrids formed using the P-1 probe demonstrates that no intervening sequences have been taken out of the region of the transcript that acted as template.

In order to map the transcription start sites precisely and to examine whether the DNA:RNA ratio in the hybridizations would affect the relative intensities of the bands, another primer extension experiment was carried out. The 194 nucleotide DdeI/FokI fragment internal to the P-1 probe (cf. Figure 1) was isolated from the replicative form of mp8-551,

labelled at the 5' ends by T4 polynucleotide kinase and [γ -³²P]ATP giving probe D-1. After strand separation, the mRNA complementary strand of D-1 was identified in an S1 nuclease assay and 10⁵ dpm of this strand was used as primer for reverse transcription after hybridization with 20 µg yeast poly(A) enriched RNA and 3 µg, 10 µg, 54 µg and 160 µg, respectively, of total yeast RNA. Figure 4A shows that the relative intensities of the extension products are unaffected by the DNA:RNA ratio in the hybridization over a 50-fold range (lanes 2, 3, 4 and 5). Furthermore, twenty distinct extension products are observed (lane 1, 20 µg poly(A) enriched RNA) corresponding to the addition of between 23 and 92 nucleotides. One strong and two faint bands with molecular weights higher than the primer are disregarded, as they are also present in lane 6 (*E. coli* tRNA). Digestion of the extension mixture with S1 nuclease revealed that all the extended DNA strands as well as the unextended primer of the DNA:RNA hybrids are S1 resistant (not shown). These results confirm that the 5' end of the *ILV1* mRNA is very heterogeneous having 20 different transcription initiation sites spanning a region of 78 nucleotides. From the DNA sequence of the *ILV1* gene (37) the positions of the 5' termini of the mRNA could be determined. This is shown in Figure 5A. The two most commonly used initiation sites (comprising about 50%) are the first A and the first C in the sequence CAAG at position -39 and -40 from the translational start. The other 18 5' termini span the region from -45 to -116. Purines do not seem to be preferred to pyrimidines as initiation points (9 purines to 11 pyrimidines).

The *ILV1* gene is transcriptionally regulated under the general amino acid control system (34). Total RNA was isolated from strain X2180-1A after growth in minimal medium plus isoleucine, valine and leucine (fully repressed) and minimal medium plus 5-methyl tryptophan (fully derepressed) and media giving intermediate degrees of derepression. The latter included minimum medium plus thiaisleucine, or plus isoleucine and valine, or plus isoleucine giving decreasing degrees of derepression. These RNAs were hybridized with the 5' end-labelled single-stranded primer D-1 de-

Figure 6. Restriction endonuclease map of the 1.55 kb EcoRI fragment covering the 3' end of the *ILV1* mRNA. The 650 bp DdeI fragment used in S1 mapping of the 3' end is indicated.

Figure 7. S1 nuclease and exonuclease VII mapping of the 3' end of the *ILV1* mRNA. The 3' end-labelled single-stranded 650 nucleotide DdeI fragment was hybridized to 80 µg total RNA from yeast (lanes 3, 5 and 6) or 10 µg of *E. coli* tRNA (lanes 2 and 4). The DNA:RNA duplexes were digested with S1 nuclease (lanes 4, 5 and 6) or exonuclease VII (lanes 2 and 3). Lane 1 is the untreated probe. The sequence of the relevant region of the *ILV1* gene is shown on the right. The brace indicate the inferred polyadenylation site. The sizes of the fragments were determined by dideoxy chain termination sequence reactions (not shown). Electrophoresis was on a 6% polyacrylamide/urea sequencing gel.

of 5 closely spaced S1 nuclease protected bands having sizes around 330 bases. Analysis on an 8% or 10% gel does not reveal any bands of similar intensity migrating faster than this cluster (not shown). We interpret these bands to reflect one 3' end since S1 nuclease often gives rise to heterogeneous products. The position of the poly(A) addition site within the *ILV1* sequence is marked by a brace in Figure 7 and Figure 5B. The DNA:RNA hybrids were also digested with exonuclease VII and the lengths of the protected fragments (Figure 7, lane 3) are very similar to those obtained with S1. Exonuclease VII gave rise to protected fragments 5-7 bases longer than those by S1. Since exonuclease VII requires a single-stranded terminus as a substrate (16), the results demonstrate the absence of noncontiguous transcribed sequences beyond the 3' terminus mapped with S1 nuclease. Lanes 2 and 4 show nuclease S1 and exonuclease VII digestions after mock hybridization of the probe to *E. coli* tRNA demonstrating that the pattern of protected bands is dependent on hybridization with yeast RNA. We conclude that the 3' terminus is located between 140 bp and 144 bp downstream from the TAA termination codon (Figure 5B).

4. DISCUSSION

We have mapped the position of the 5' and 3' ends of the *ILV1* mRNA. THURIAUX et al. (65) made a detailed genetic analysis of *ILV1* and on the basis of the distribution of complementing nonsense alleles it was concluded that *ILV1* translation starts at the centromer proximal end of the locus. Using this information the map of the *ILV1* region shown in Figure 1 can be oriented, i.e. the *Sall* site is towards the telomere on the right arm of chromosome V.

The 5' end of the *ILV1* mRNA displays a high degree of heterogeneity, revealing 20 different transcription initiation sites spanning a region of 78 nucleotides. No transcription initiates downstream from position -39 supporting our previous suggestion that the ATG at +1 is in fact the point of translation initiation (37). Also, the full 1,728 bp open reading frame must be translated, since no intervening sequences have been found in this region. Whether multiple 5'

ends reflect alternate sites for transcription initiation or mRNA processing has not been established. The fact that the sites determined by S1 nuclease and primer extension are identical rules out artifacts of these procedures such as local low stability of the DNA:RNA hybrids. However, until this question has been investigated we shall for the sake of simplicity presume that the 5' termini determined by primer extension correspond to sites for transcription initiation. The two most prevalent start sites are the first A and the first C in the sequence CAAG at position -39 and -40 from the coding region. This sequence is present in the promoter of several other yeast genes (14, 19, 24, 32, 52) and it has been proposed to be a yeast mRNA cap site (52). Previously, this cap site has not been found in genes coding for amino acid biosynthetic enzymes. DOBSON et al. (19) have suggested that the CAAG sequence, together with a pyrimidine-rich block (CT-block) located about 10 bp upstream constitute a structure involved in high efficiency gene expression in yeast. These sequences and their relative positions are conserved amongst highly expressed genes (e.g. genes encoding glycolytic enzymes) (19). In front of the *ILV1* gene we have noted a CT-rich region (at position -53 to -61, see Figure 5A) followed, 12 bp downstream, by the CAAG sequence. Since threonine deaminase constitutes about 0.1% of total cell protein, i.e. 50 times lower than e.g. phosphoglycerate kinase, it appears that the CT-CAAG structure alone is not sufficient for high gene expression in yeast.

Another feature of the *ILV1* mRNA is that all the transcription initiation sites map within the 93 bp open translational reading frame (ORF) starting at position -117 and within one half of the large imperfect inverted repeat (-185 to -35) noted earlier (37). If this ORF is transcribed, the 3' end of the RNA must be located very close to the TAA stop codon (at -29), since reverse transcription employing the D-1 probe (-21 to +173) showed that all extension products ended inside the ORF (cf. Figure 5A). Upstream open reading frames are not uncommon to amino acid biosynthetic genes in yeast (3, 20, 28, 67). Although the ORF in front of *HIS4* is transcribed (25), a deletion analysis has shown

that it is not required either in cis or trans for the regulation of *HIS4* by the general control system (38).

Amongst other yeast genes described so far, only the *HIS1* mRNA (28) shows a similar degree of 5' heterogeneity as *ILV1*. Genes such as *HIS4* (20), *HIS3* (64), *PGK* (19) and the gene for actin (24) have a single site for transcriptional initiation. Other genes such as *TRP2* (68), *TRP3* (1, 68) *CYC1* (22) and *ADCI* (5), display 4-7 different 5' termini.

Eukaryotic mRNA initiation sites are preceded by an AT-rich region, the TATA box, with the canonical sequence TATA₁A₁ (11) recognized by RNA polymerase II. In contrast to higher eukaryotes, the distance between the TATA box and the cap site in yeast varies considerably (58). In front of *ILV1* there are three such sequences, TATGAAA at -323, GCATAAAA at -166 and TATCAAA at -146 being 207 bp, 50 bp and 30 bp upstream from the RNA initiation site for the longest transcript. In the case of the *HIS1* mRNA (28) every identified 5' end can be associated with a TATA like sequence that lies 25-45 bp upstream, whereas this is not possible in *ILV1*. However, 5' flanking regions of genes having a single cap site also possess an abundance of TATA like sequences (20, 64). In the case of SV40 early genes (7), rabbit β -globin (26) and the *MAT α* gene (59) the lack of a TATA box does not eliminate transcription, but instead initiation occurs at multiple sites. Changing the β -globin TATA box from GCATAAAA to GCATGTAA led to a 5-fold reduction in transcription initiation efficiency and produced heterogeneous transcription initiation (18). Interestingly, the *ILV1* AT-rich sequence at -166 is identical to the rabbit β -globin TATA box. Further experimentation is required to determine the nature of the sequences involved in selecting the transcription initiation sites of *ILV1*.

Another sequence thought to be important for transcription initiation in eukaryotes is the CAAT box with the consensus sequence GC₂CAATCT (8). Several genes have this sequence about 80 bp upstream the initiation of RNA synthesis. With G(T)TCAAT(T)T *ILV1* has an easily recognizable CAAT box (7/9) at -245, but its position 129 bp upstream from

the closest RNA start site does not fit the consensus pattern.

The steady state level of the *ILV1* mRNA is elevated in response to tryptophan starvation, showing that the gene is under general amino acid control (34). A transcriptional response has also been demonstrated for a number of other genes which respond to general control (1, 28, 60, 63, 67). The 5' flanking regions of *HIS3* and *HIS4* contain short sequences that mediate general control. These sequences lie in *HIS3* at about -40 and -100 upstream to the 5' terminus of the mRNA (about -80 and -140 relative to the ATG) (61) and at -136, -180 and -192 in the corresponding *HIS4* region (at -198, -242 and -254 relative to the ATG) (21). That the sequence TGACT, contained in the above regions, is the functional core sequence for the general control is indicated by base substitution revertants of partial deletions in *HIS4* which recreate the sequence and restore general amino acid control (18). Furthermore, a synthetic oligonucleotide with the -136 *HIS4* general control sequence TGACTCACG was substituted for the upstream regulatory region (the UAS containing 450 bp *XhoI* fragment) of the *CYC1* gene and promoted standard *CYC1* mRNA but now subject to general amino acid control (30). The TGACT containing elements are likewise found in DNA upstream from the coding regions of *ARG4* (6), *HIS1* (28), *TRP5* (67), *TRP2* (68) and *TRP3* (1, 68) all of which are under general amino acid control. It is presumed that the *GCN4* product interacts with these sites to activate transcription under conditions of amino acid starvation (29). In the *ILV1* upstream DNA the element TGACT is found at positions -22, -132 and -307, once in the orientation in which it has been described in *HIS1*, *HIS3*, *HIS4*, *ARG4*, *TRP5*, *TRP2* and *TRP3* (1, 6, 28, 68) and twice in the reverse orientation (37). The element at -22 is located within the region transcribed into mRNA and is therefore probably not to be considered. The element at -132 is located only 16 bp 5' to the first cap site and only 14 bp 3' to the putative TATA box at -146. In contrast, the third sequence is 199 bp upstream the cap sites and 16 bp 3' to the TATA box at -323. The positions of the TGACT elements in relation to the TATA boxes and

the mRNA cap sites are different from the other investigated genes and may be one reason for the relatively weak derepression response of *ILV1* (34). As is the case in *HIS1* (28) the 5' heterogeneity does not seem to reflect mRNA classes which are independently regulated since the various mRNA species increase to the same extent in response to different degrees of amino acid starvation.

The 3' end of the *ILV1* mRNA was mapped using nuclease S1 and exonuclease VII. In contrast to the heterogeneous 5' end, only one 3' end was deduced. It is located between positions +1871 and +1875, resulting in mRNA containing between 140 and 144 nucleotides of downstream untranslated sequence. Transcription continues beyond the poly(A) site in some higher eukaryotic genes (47) and termination of transcription and polyadenylation of the resulting mRNA are presumably two functionally distinct but perhaps coordinate events. In higher eukaryotes, the sequence AAUAAA has been found to precede the poly(A) tail of most messages (47). This sequence is probably not involved in yeast polyadenylation, as it is seldomly found in yeast messages (69), and it is not present at the 3' end of *ILV1* mRNA either. ZARET and SHERMAN (69) have proposed that polyadenylation may be coupled to transcription termination by some component of RNA polymerase II. They suggested the sequence TAGT or TATGT as a component of a tripartite termination/polyadenylation signal in yeast. In *ILV1* the structure TAG---TATGT---TTT is found between nucleotides +1804 and +1832 and located about 40 bp before the 3' mRNA end. A second potential yeast polyadenylation/termination sequence TATTTATA at position +1828 differs only at one position from the element TTTTATA suggested by HENIKOFF et al. (27) to be important for the maturation of the *Drosophila ADE8* message in yeast.

In conclusion, we have characterized *ILV1* transcription and discussed putative sequences important for regulation and transcription of the gene. It will be of interest to compare the flanking regions of the gene with the corresponding DNAs of the functionally homologous, but structurally different *ILV1* gene present in *S. carlsbergensis* (48).

ACKNOWLEDGEMENTS

We thank G. BANK for excellent technical assistance, C. POULSEN for discussion and valuable suggestions and D. VON WETTSTEIN for critical reading of the manuscript. This work was supported by grants 16-3557.H-699 from the Danish National Research Council for Technical Sciences, and 133/001-84154, from Teknologirådet of the Danish Ministry of Industry to D. VON WETTSTEIN.

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Accepted by H. KLENOW