Temperature-sensitive eIF5A Mutant Accumulates Transcripts Targeted to the Nonsense-mediated Decay Pathway

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The highly conserved protein eIF5A found in Archaea and all eukaryotes uniquely contains the posttranslationally formed amino acid hypusine. Despite being essential the functions of this protein and its modification remain unclear. To gain more insight into these functions temperature-sensitive mutants of the human eIF5A1 were characterized in the yeast Saccharomyces cerevisiae. Expression of the point mutated form V81G in a ΔeIF5A strain of yeast led to a strongly temperature-sensitive phenotype and to a significantly reduced protein level at restrictive temperature. The mutant showed accumulation of a subset of mRNAs that was also observed in nonsense-mediated decay (NMD)-deficient yeast strains. After short incubation at restrictive temperature the mutant exhibited increased half-lives of the intron containing CYH2 pre-mRNA and mature transcripts of NMD-dependent genes. Reduced telomere silencing and shortening was detected in the V81G mutant further supporting similarities to NMD-deficient strains. Our data suggest that eIF5A mediates important cellular processes like cell viability and senescence through its effects on the stability of certain mRNAs.

The cellular physiology of mRNA processing, transport, localization, and turnover is central to the process of gene expression at the posttranscriptional level. Increasing evidence has been found for a close connection between mRNA degradation processes and the steps of translation. The highly conserved hypusine-containing protein eIF5A has been implicated in both of these aspects of RNA metabolism; however, its precise cellular function is not yet fully understood.

Hypusine formation is a two-step enzymatic reaction catalyzed by deoxyhypusine synthase and then deoxyhypusine hydroxylase (1). The disruption of genes encoding either eIF5A or deoxyhypusine synthase in yeast leads to a lethal phenotype (2) demonstrating that the deoxyhypusine residue is essential for the function of eIF5A and thus for proliferation and cell survival. The genome of Saccharomyces cerevisiae contains two HYP genes (HYP1, alias TIF51B or ANB1, and HYP2, alias TIF51A) coding for eIF5A. These genes are differentially expressed under aerobic and anaerobic conditions (3) and share an identity of 90%. HYP genes of other higher eukaryotes, e.g., one of the two human HYP genes (encoding EIF5A1), can functionally replace these yeast-specific genes (4, 5).

The eIF5A protein was first isolated from rabbit reticulocyte lysate ribosomes and classified as a translation initiation factor because in vitro the protein enhanced the building reaction of the first peptide bond measured as the yield of methionine-puromycin (6). However, cell fractionation revealed that only a small fraction of the protein associated with ribosomes (7). Moreover depletion or inactivation of the protein in S. cerevisiae reduced the global protein synthesis rate by only 30% (8, 9). Thus, the role of eIF5A as a translation initiation factor remains to be confirmed.

eIF5A is an RNA-binding protein (10), and it was hypothesized to regulate the translation of a subset of mRNAs that are needed for G1/S cell cycle progression because agents blocking deoxyhypusine hydroxylase (11) or deoxyhypusine synthase (12, 13) induced a cell cycle arrest at the G1/S boundary in several mammalian cell types. These results were supported by the G1 arrest at 37 °C of yeast cells expressing a temperature-sensitive point mutated form of Tif51A (14).

The finding that eIF5A is a cellular cofactor of human immunodeficiency virus type 1 Rev and human T-cell lymphotrophic virus type 1 Rex transactivator proteins in mRNA export and the interaction of eIF5A with the general exportin Crm1p for nuclear export signal-containing proteins in higher eukaryotes suggested it played a role in nucleoplasmatic shuttling of mRNA (15–17). However, although the protein was found to be located in the nucleus and the cytosol of COS-7 cells (18), active shuttling between both compartments could not be confirmed. Also a direct interaction between Rev and eIF5A has not been shown (19). In addition yeast strains expressing temperature-sensitive mutants of Crm1p did not show a mislocalization of eIF5A to the nucleus (20). Therefore the nuclear export hypothesis has been questioned.

The implication of eIF5A in mRNA degradation initially came from studies of a temperature-sensitive HYP2 mutant (ts1159) that showed an accumulation and a strongly prolonged

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‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Table S1.

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¶ The in vivo assays were performed in the gene expression and hybridization array data repository of the National Center for Biotechnology Information (GEO, http://www.ncbi.nlm.nih.gov/geo/, accession number GSE5290).
half-life of unspliced CYH2 mRNA (9). The genetic background of
this strain, however, reveals a functional HYP1 introducing
the possibility that it might affect the observed phenotypic
effects.

Two studies revealed a connection between eIF5A and senes-
cence processes showing a transcriptional up-regulation of
dehydropyrimidine synthase and eIF5A in aging tomato plants (21,
22). However, in mammalian IMR-90 cells senescence led to a
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Using yeast as a model system in which both native HYP
genes were disrupted we describe the effects of expressing the
point mutation V81G in the human hypusine-containing pro-
tein eIF5A1. We observed strong temperature sensitivities in
these yeast strains. Expression of the mutant protein resulted in
the accumulation of nonsense-containing RNAs, which are
known to be specifically degraded by the polyadenylation-inde-
dependent 5′–3′ mRNA decay pathway (nonsense-mediated
decay (NMD)2). Additionally elongated half-lives of selected
NMD transcripts and shortened telomeres were observed.

These results suggest a functional connection of eIF5A with the
NMD machinery that is coupled to translation initiation and reinforce the notion that the protein might influence essential
cellular functions via its role in RNA processing.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Plasmids, and Growth Conditions**—Yeast cells
were grown either on semisynthetic medium with 2% galactose
or 2% glucose or on yeast-peptone-dextrose medium and yeast-
peptone-galactose medium (24). The plasmid shuffle with and
without 5-fluoroorotic acid as selective agent was performed as
described previously (25). *S. cerevisiae* strains used in this study
are listed in Table 1. The disruption of the HYP1 gene was
performed with the haploid strain WDH6–9[ YepHyp2] (3)
bearing a disruption of HYP2 by a 2.22-kb LEU2 fragment. The
HYP1 gene (cloned as a 1.94-kb EcoRI-BamHI fragment in a
plasmid derived from pBluescript; Stratagene) was disrupted by
insertion of a 1.6-kb Smal-AatII fragment carrying the
TRP1 marker gene into the single Sall site of the HYP1 coding region.

From the resulting vector a linear 3.6-kb EcoRI-BamHI frag-
ment was used for homologous recombination according to
Rothstein (26). Correct integration was verified by Southern
analysis. Isolation of high molecular weight genomic yeast
dNA, cloning, and in vitro mutagenesis of DNA were per-
duced previously (24, 31). PCR amplification was
used to clone the coding regions of different wild-type and
point mutated HYP genes according to the properties of multi-
ple cloning sides in the target vectors. The plasmids used and
produced for this study are listed in Table 2. The constructs for
HYP expression either harbored the coding sequence of the
HYP2 gene (*S. cerevisiae*) or the cDNA of the human HYP1 gene
EIF5A1. Transformation of yeast strains was performed using the

**Anti-eIF5A Western Blotting**—To determine eIF5A levels in
V81G mutant strains, cells were grown to an A600 of 1 at 25 °C
or shifted to 37 °C and incubated at this temperature for a fur-
ther 6 h. SDS-PAGE and immunoblotting were performed as
described previously (33) using a 1:10,000 dilution of polyclonal
antisera against human EIF5A1 and enhanced chemilumi-
nescence detection (GE Healthcare).

**MTT Cell Viability Test**—An MTT viability assay of mutant and
wild-type yeast cells was performed as described previously (34).

**Terminal Deoxynucleotidyltransferase-mediated dUTP Nick
End Labeling (TUNEL)**—The TUNEL assay and appropriate
cell preparations were performed as described previously (35).

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**TABLE 1**

| Strain             | Genotype                                                                 | Source or Ref. |
|--------------------|--------------------------------------------------------------------------|----------------|
| W303-1MATa         | MATa, leu2-3, 112, ura3-1, 112, his3-11, 15, trp1-1, 15, ade2-1, can1-100 | 26             |
| W303Δh1h2          | MATa, hyp1-1TRP1, hyp2-1LEU2, HYP2, YEp (URA3) else isogenic to W303-1MATa | This study     |
| WDHyp2-Gal         | MATa, hyp1-1TRP1, hyp2-1LEU2, HYP2, pRS | This study     |
| WDH(hum)Gal        | MATa, hyp1-1TRP1, hyp2-1LEU2, EIF5A1, pRS | This study     |
| WDHum2-1Gal        | MATa, hyp1-1TRP1, hyp2-1LEU2, EIF5A1, V81G, pRS | This study     |
| PLY118             | MATa, ura3-5A4-S1A3 ura3-5A4-S1A3 ura3-5A4-S1A3 leu2-3;112 2 his4-38 | 27             |

**TABLE 2**

| Plasmid Description | Ref. |
|---------------------|------|
| Yepl52              |      |
| Yepl52              |      |
| YEp35S22            |      |
| YEp35S2            |      |
| pRS315              |      |
| pRS315              |      |
| pRS313              |      |
| pRS316              |      |
| pRS313-HYP2         |      |
| pRS313-HYP2         |      |
| pRS313-EIF5A1       |      |
| pRS313-EIF5A1G81    |      |
| pAA79               |      |
| pCM189              |      |
| pCM189              |      |
| pCM189              |      |
| pCM189              |      |

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2 The abbreviations used are: NMD, nonsense-mediated decay; FITC, fluores-
cein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogen-
ase; HDOA, high density oligonucleotide array; LTR, long terminal repeat;
MTR, 3′-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; ORF,
open reading frame; ts, temperature-sensitive; TUNEL, terminal deoxynucleo-
tidyltransferase-mediated dUTP nick end labeling; Ty, transposon yeast; fmk,
fluoromethyl ketone; PD, population doubling.
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using the In Situ Cell Death Detection kit (Roche Applied Science). Coverslips were mounted with a drop of 5% n-propyl gallate (Sigma) in glycerol (100%). Slides were analyzed by fluorescence microscopy.

Measurement of Caspase Activation—In vivo staining of caspase activity by flow cytometric analysis was performed as described previously (36) using FITC-VAD-fmk (CaspACE, Promega) and a FACScalibur system (BD Biosciences). Wild-type cells treated with 3 mM H₂O₂ served as a positive control.

Generation of Gene Expression Datasets by Microarray Analysis—For high density oligonucleotide array (HDOA) analysis total RNA was isolated using the hot phenol method (37) from cultures grown at 25 °C reaching early logarithmic phase. For each experiment RNA was independently isolated from four isogenic yeast strains of the wild type and the mutant (strains WDH(hum)Gal and WDHG₆₈(Gal), and in vitro transcriptions and hybridizations were performed. Thus, a total of eight chips were included in the analysis (4 replicates × 2 datasets for the wild type and the mutant V81G). RNA was further purified with RNasea columns (Qiagen). In brief 30 µg of total RNA were subjected to a cDNA synthesis reaction to make the first strand using an oligo(dT) primer with a T7 promoter sequence added to the 5′-end. After synthesis of the second strand, double-stranded cDNA was purified by phenol/chloroform extraction, precipitated, and resuspended in nuclease-free water. Biotin-labeled cRNA was made by in vitro transcription using the High Yield Transcription kit (ENZO Diagnostics). The resulting cRNA was fragmented at 94 °C for 35 min in buffer A (40 mM Tris acetate, 100 mM KOAc, and 30 mM Mg(OAc)₂). Affymetrix yeast S98 GeneChip® arrays were hybridized, washed, stained, and scanned according to the manufacturer’s specifications. Scanned raw data images were processed with Affymetrix GeneChip Version 3.2 software.

Normalization and Statistical Analysis of Hybridization Data—Data from the arrays was normalized, and expression values based on an additive model were calculated according to the method of Irizarry et al. (38). Differentially expressed genes were identified by the permutation-based method of Tusher et al. (39). Briefly to control for multiple testing, a false discovery rate (40) was calculated as the percentage of genes falsely detected as differentially expressed among all genes detected as differentially expressed. The q value is the lowest false discovery rate at which the gene is called significant. Significant genes were identified if they exhibited the lowest q value computed by SAM (“Significance Analysis of Microarrays”) software. Detailed information regarding the analysis performed on the microarray data can be found in the supplemental data section. Primary array datasets were published in the gene expression and hybridization array data repository of the National Center for Biotechnology Information (Gene Expression Omnibus (GEO), www.ncbi.nlm.nih.gov/geo/, accession number GSE5290). The transcriptomic profiles of V81G with NMD-deficient yeast were compared with the original .cel files published by He et al. (41) and are available at www.ebi.ac.uk/miameexp/ under accession numbers E-MEXP-26 and E-MEXP-27. These datasets were analyzed in exactly the same way as the microarray data produced for this study.

Northern Blotting and Determination of mRNA Half-lives—Yeast strains were cultured until they entered early log phase at either 25 or 37 °C. Total RNA was isolated as before (37). Northern blotting was carried out as described previously (42). RNA was detected by hybridization to digoxigenin-labeled DNA probes of about 350 bp in length that were prepared by PCR using yeast genomic template DNA. After hybridization and stringent washing signal detection was performed using anti-digoxigenin antiserum (Roche Applied Science). Quantification of RNA bands was performed using densitometry. The stability of selected mRNAs was determined according to Parker et al. (43). Briefly 200 µl of mutant and wild-type yeast strains were grown to A₆₀₀ = 0.7 and incubated for 1 h at either 25 or 37 °C. Next transcription was inhibited by the addition of thiolutin (20 µg/ml), and subsequent samples were taken at 0-, 4-, 8-, 16-, 32-, and 64-min intervals and frozen quickly in a dry ice-ethanol bath. Subsequently total RNA was isolated, and 10 µg of RNA/lane was analyzed by Northern blotting as described above.

Quantitative Real Time (Reverse Transcriptional) PCR—Gene-specific primers were designed with the PrimerExpress software (Applied Biosystems). The antisense primers were used for reverse transcription with avian myeloblastosis virus reverse transcriptase (Promega) according to the manufacturer’s instructions and using 2 µg of total RNA. Real time PCR was performed using SYBR® Green PCR Master Mix and a GeneAmp5700 sequence detection system (Applied Biosystems) according to the protocols of the manufacturer. Relative expression levels were calculated, and PCR efficiencies were determined as described previously by Pfaffl (44) and Ramakers et al. (45), respectively, using ACT1 expression for normalization. For each RNA preparation tested a minimum of three independent real time (reverse transcriptional) PCR experiments were conducted.

Determination of Telomere Length by Southern Analysis—Telomere lengths were determined as described previously (46). Briefly a probe containing a telomere repeat sequence was prepared by 5′-end labeling of the oligonucleotide Tel-Rep4 (CACCACAACCCCACACCCACACCCCCACACCA-CAC) with digoxigenin. The sequence was identical to 2.5 repeats of the telomere template sequence from the chromosome 8 analogue to TLC1. Total genomic DNA was digested with PstI, and Southern blotting was performed as before. Signal development was achieved by using the DIG (digoxigenin) Luminescent Detection kit (Roche Applied Science) according to the manufacturer’s instructions.

RESULTS

Isolation of the Temperature-sensitive Point Mutant V81G—To study the relationship between structure and function in the human eIF5A1 hypusine-containing protein point mutations were made throughout the entire length by exchanging phylogenetically conserved residues with related amino acids. A yeast strain was constructed (W303Δh1h2) in which both the genes HYP2 and HYP1 (AN81) encoding for the hypusine-containing protein were disrupted (3). The strain harbored a 2µ-LIRASplasmid-borne wild-type copy of the genomic HYP2, thus complementing the otherwise lethal phenotype. The constructs
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containing the point mutations to be tested were cloned into pRS313 containing the GAL promotor and transformed into the W303ah1h2 cells. The exchange of the wild type for a mutated allele was performed by selective plasmid shuffling using fluoroorotic acid. Because hypusine is essential, cell death occurred if the mutant allele cloned in pRS313 was incapable of complementing the gene function of the wild type. This counterselection was used to screen all mutations in the human HYP homologue EFS5A1.

The expression of certain mutated alleles resulted in temperature-sensitive yeast strains. The strongest temperature sensitivity was observed by the substitution of the valine residue at position 81 (Fig. 1). Proliferation of cells at the restrictive temperature was stopped after only one round of doubling when valine at position 81 was mutated to a glycine (V81G). In comparison with wild type the growth of the mutated strain at the permissive temperature was decelerated. Retransformation of the mutant strains with a single copy plasmid carrying wild-type human EFS5A1 completely restored the temperature sensitivity (Fig. 1C) indicating that it was not a recessive mutation. Several independent transformations and subsequent 5-fluoroorotic acid selections reproducibly showed the pronounced phenotype of the mutant V81G. These mutated strains were chosen for the subsequent phenotypic characterization experiments.

eIF5A Protein Levels and Viability at 37 °C—Complete cell lysates were prepared from wild-type or mutant cells after incubation at permissive or restrictive temperatures (Fig. 2). The eIF5A protein levels in each case were determined by Western blotting. At 25 °C the mutated protein was expressed to nearly the same amount as the wild type. Within 60 min after heat shock induction the level of the eIF5A (V81G) protein declined more than 5-fold. A further 5-h incubation at 37 °C did not result in further changes in the protein level.

The viability of V81G-expressing cells at both temperatures was checked with an MTT assay (Fig. 3). Independently isolated strains of the mutant V81G and the corresponding wild-type strains that were in early logarithmic growth phase were tested at both the restrictive and the permissive temperatures. Fig. 3 shows that the formation of blue formazan from the activity of dehydrogenases was similar for the wild-type cultures grown at either the restrictive or the permissive temperature. Slight differences were observed between wild-type cultures at 37 °C grown at both restrictive and permissive temperatures. The viability of V81G-expressing cells was lower than that of the wild type, but the difference was not statistically significant. However, even at the permissive temperature, V81G, although capable of growth, displayed a 50% reduction in viability when compared with the wild type. After a 6-h incubation at 37 °C the viability was further decreased.

To determine whether the mutant’s loss of cell viability was due to apoptotic cell death the TUNEL assay was performed, and the results are shown in Fig. 4B. No fluorescence was detected in the control cells expressing wild-type eIF5A regardless of the growth conditions. However, addition of 3 mM H2O2, and incubation for 6 h at 37 °C rendered the cells positive for DNA breaks, which served as a positive control. At the permissive temperature (25 °C) the V81G mutant did not show any fluorescent nuclei. In contrast, after 4 h at the restrictive temperature (37 °C) clear fluorescence in the nuclei was visible and increased with longer incubation times.

To determine whether endogenous caspases were activated in the mutant as a result of the induction of apoptosis a flow cytometric assay was performed. Yeast cells were incubated in vivo with FITC-labeled VAD-fmk (FITC-VAD-fmk), which specifically binds to the reactive center of active caspases in eukaryotic cells (36). Fig. 4Bii shows that incubation of mutant cultures at 37 °C for 6 h activated caspases in 33% of the counted cells. An equal fraction of apoptotic cells was detected in wild-type cultures in which apoptosis was induced by treatment with 3 mM H2O2. In contrast the V81G mutant strain showed no
caspase-activated cells in cultures that had been incubated for a shorter time (3 h) at a higher temperature or at 25 °C. Propidium iodide fluorescence can be used to detect cells that have died independently of necrotic or apoptotic triggers. As nearly all propidium iodide-positive cells also showed FITC-VAD-fmk fluorescence (accumulation of cells in the upper right region R1 of the dot blot, Fig. 4 Ai cell death in these cells occurs by apoptosis rather than by necrosis. In summary the results show that incubation at 37 °C but not at 25 °C led to phenotypic effects related to programmed cell death in the V81G strain. Due to the V81G growth characteristics, all subsequent experiments were performed with the V81G strain that had been incubated either at 25 °C or at 37 °C less than 1 h.

**Genome-wide Transcriptome Analysis of the Mutant V81G Strain**—To identify transcripts regulated by eIF5A, RNA expression profiles of wild-type and V81G strains cultured at the permissive temperature were analyzed. HDOAs were used that covered the whole yeast genome (47). On the array, 9275 probe sets encoding 7839 yeast genes were present of which that covered the whole yeast genome (47). On the array, 9275 probe sets encoding 7839 yeast genes were present of which 345 accumulating NMD transcripts including putative or non-annotated ORFs postulated by the yeast genome sequencing project or serial analysis of gene expression.

cDNA probes derived from total RNA preparations were obtained from four isogenic, haploid strains from each of the wild type and the mutant. This ensured that changes in the transcriptome could be attributed to the impaired *HYP* function of V81G rather than to differences in the genetic background. Therefore, two groups of four datasets (per wild-type and mutant strains) were generated after hybridization to the HDOAs. The robust multichip average procedure (“Experimental Procedures”) could be used for the normalization of all samples because we showed that the quantification of the absolute RNA amounts that were spiked prior to cDNA synthesis were found to be identical. Transcripts were classified as differentially expressed if they could be assigned to the lowest q value of 0.08796% indicating a high level of significance.

In this way the pairwise comparison of the transcriptomes of the V81G and wild-type strains resulted in 604 differentially expressed transcripts (Table 3). 415 (421 probe sets) showed up-regulation, and 188 (205 probe sets) showed down-regulation. These two transcript groups served as the basis for the following evaluations. A functional classification of up- and down-regulated genes and ORFs in V81G mutant strains (according to Ref. 48) can be found in the supplemental data to this study (see supplemental Fig. S1). The temperature shift from 25 to 37 °C and a 3-h period of incubation at high temperature barely had any effect on the distribution of gene functions (data not shown).

**Partial Abrogation of the Telomere Position Effect and Enrichment of Ty Retrotransposon RNAs**—345 accumulating ORFs in V81G (without those found by serial analysis of gene expression) were analyzed with regard to their chromosomal location. We found that 29.3% of the ORFs in the yeast genome (119 of 406) were within a 27-kb region of the telomeres. These subtelomeric ORFs were up-regulated in the mutant strains. In contrast only 3.4% of the non-telomERICally encoded ORFs of the yeast transcriptome (199 of 5863 ORFs; without Ty and LTR elements) were up-regulated. Furthermore a connection between the level of enrichment and the number of telomeric transcripts was seen. Of the 345 ORFs the greatest enrichment was observed for the ORFs closest to the telomeres (subtelomeric), whereas the bulk of the remaining ORFs that showed less enrichment were found further from the telomeres (see Fig. 5).

Genes located nearby chromosomal telomeres are transcriptionally repressed in *S. cerevisiae*. This chromosomal area contains a lower density of ORFs than other regions. The phenomenon was named the telomere positioning effect (49). Normally the telomere positioning effect rapidly decreases as the distance between an ORF and the telomere increases. At every telomere a number of ORFs were found for which transcriptional activity was not altered when compared with the wild type. Thus, referring to the data, the telomere positioning effect in the mutant V81G was reduced but was not completely abolished compared with the wild type.

Retrotransposons and their controlling elements represent 0.9% of the entire ORFs in the whole yeast genome.
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When we compared this to the pool of transcripts that were increased in the V81G strain we found that this number increased to 10% of the total transcripts (see supplemental Fig. S1). The number of enriched Ty retrotransposonal transcripts including two Ty4 and two Ty3 full-length RNAs and about 40 transcripts coding for LTR elements of all five kinds of retrotransposons (Ty1–5) are shown in Fig. 5 (for details see supplemental data file Array-Data-S4). Of these most LTRs originaited from Ty1 and Ty4 elements. Interestingly we observed a significant portion of the accumulating transcripts (15%) near the Ty retrotransposons or LTR sequences remaining from former retrotransposition processes. Especially interesting was a position upstream of the 5′-end of a Ty-LTR that led to accumulation of the RNA of the corresponding adjacent ORF. This points to eIF5A playing a role in processes involving retrotransposons.

Significant Overlap of the V81G-accumulating Transcriptome with Those of Strains Deficient in 5′−3′ mRNA Degradation—We reproducibly found a 4-fold up-regulation of the LIPF2 mRNA. The corresponding protein together with Upf1p and Upfs3p function as trans-acting factors of the mRNA NMD pathway. This pathway also includes the mRNA decapping enzymes DCP1 and DCP2 and the 5′-exonuclease XRN1. Of all genes belonging to the NMD pathway only the LIPF2 tran-

### Table 3

| Strain                  | q^v       | Increases | Decreases | Both, total |
|-------------------------|-----------|-----------|-----------|-------------|
|                         | %         | ORFs      | Further elements | ORFs | Further ORFs | ORFs | Further ORFs | Both, total |
| eIF5A-V81G-ts           | 0.08796   | 298       | 32         | 85         | 415        | 163  | 4           | 21          | 188 | 604 |
| upf1A                   | 0.07775   | 380       | 39         | 105        | 524        | 38   | 1           | 2           | 41   | 565 |
| upf2A                   | 0.06749   | 376       | 36         | 90         | 502        | 48   | 6           | 9           | 63   | 565 |
| upf3A                   | 0.05349   | 476       | 39         | 121        | 636        | 69   | 13          | 10          | 92   | 728 |
| upf23A                  | 0.01432   | 664       | 46         | 133        | 843        | 278  | 28          | 35          | 341  | 1184 |
| xrn1A                   | 0.02426   | 640       | 41         | 116        | 797        | 361  | 34          | 38          | 433  | 1230 |

^a The q value computed by the SAM (Significance Analysis of Microarrays) software represents the threshold of significance for finding accumulated or decreased transcripts.

^b ORFs indicates transcripts encoded by protein-coding genes on the chromosomes.

^c Further elements represents transcripts encoded by genes of rRNAs, tRNAs, small RNAs, and RNAs encoded by transposable elements and their LTRs.

^d Further ORFs, non-annotated or putative ORFs identified by the genome sequence project and by serial analysis of gene expression as well as genes of DNA plasmids or those encoded on the mitochondrial genome.

^e upf23A is the combination of transcriptomic profiles of each of the three LIPF genes.
scripts showed a changed mRNA level in comparison with the wild type.

Two genome-wide studies of transcriptomic profiles from UPF123, DCP1, and XRN1 knock-out yeast strains have been performed (41, 50). The transcriptomes of the elf5A-ts mutant were compared with NMD-deficient strains using primary microarray data published by He et al. (41) by performing the same normalization and statistical analysis procedures with all hybridization datasets.

Knock-out of any of the UPF genes resulted in the up-regulation of 705 ORF mRNAs (see Fig. 6, A and B, upper left circles) including Ty retrotransposons and their LTRs. On average 139 transcripts were found to be depleted (Fig. 6C, upper left circle). The dcp1Δ and xrn1Δ strains showed a higher number of regulated ORFs (Table 3). 843 ORFs in the dcp1Δ and 797 in the xrn1Δ strains accumulated.

Among the up-regulated RNAs in the V81G strain numerous transcripts overlapped with those up-regulated in strains bearing knock-outs of components in either the NMD or 5′–3′ mRNA degradation pathway, respectively (Fig. 6A). 243 transcripts (59%) of the on average 415 increasing ORFs in the mutant were also up-regulated within the pool of up-regulated transcripts of upf123Δ strains. Compared with those transcripts up-regulated in xrn1Δ/dcp1Δ strains the overlap was about 10% lower (208 ORFs in comparison with dcp1Δ and 194 ORFs in comparison with xrn1Δ strains, representing 50 and 47% of the enriched transcriptome of V81G). Examination of the down-regulated transcripts in NMD and hyp-deficient strains showed no significant overlap (Fig. 6C).

**Determination of mRNA Steady States and Decay Rates**—To validate the obtained microarray data and to find evidence that the accumulation of mRNAs in V81G was due to a direct mRNA decay defect in the mutant and not due to indirect effects that influence mRNA levels, mRNA steady states and decay rates of selected transcripts were measured.

The relative levels of nine transcripts found to accumulate in the V81G strain were analyzed by Northern blotting (Fig. 7A) and real time (reverse transcriptional) PCR (Fig. 7B) after the cells were cultivated at 25 °C. These transcripts were shown to be up-regulated thus supporting the microarray results. Two additional transcripts of factors that function in telomere length control (EST1 and S7N1) were quantified and also found to be up-regulated in the mutant. Furthermore mRNA stability was determined for five transcripts (Fig. 7C). Specifically the intron containing CYH2 pre-mRNA, a direct target of NMD, showed only a slightly prolonged half-life in the mutant at the permissive temperature compared with the wild type. After a shift to 37 °C and a further incubation for 1 h the transcript was stabilized with a decay rate decreasing by a factor of 15 (Fig. 7, left).

The ARN1 and BSC4 transcripts accumulated similarly in V81G and NMD knock-out strains (41). 3–5-fold increased half-lives were determined for both transcripts at 37 °C in the mutant strain. Based on the microarray data the transcripts of the 40 S ribosomal protein S5 (RP5) and GAPDH2 (TDH2) served as negative controls. Both transcripts showed no differences in RNA half-lives (Fig. 7C) between the wild-type and V81G strains.

**Shortening of Telomeres in V81G Mutant Strains**—In the elf5A mutant several mRNA species of genes with functions in the metabolism of telomeres, including the telomerase catalytic subunit encoded by EST2, were significantly up-regu-

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**FIGURE 5.** Distribution of telomeric and non-telomeric ORFs within the group of up-regulated transcripts in V81G strains. The bar code distribution (divided into four parts) represents 345 up-regulated ORFs in the mutant sorted in a descending order for their mean -fold change factors. The number of telomeric ORFs increases with increasing -fold change factors (indicated by the punctured trend line). In accordance most transcripts showing slight increases are not positioned near telomeres of chromosomes. Also information about the distribution and number of transcripts of Ty retrotransposons (including their LTR elements) and ORFs being located near Ty elements and Ty-LTRs on the chromosomes is included.

**FIGURE 6.** Overlap between the transcriptomic datasets of the HYP mutant V81G and nmdΔ yeast strains. A and B, overlap of elevated transcriptomes. The **upper left sections** named upf123Δ represent the combined number of elevated ORFs in strains carrying disruptions in one, two, or all three UPF genes (41). The number of elevated transcripts in xrn1Δ or dcp1Δ strains is contained in the **upper right sections**. The overlaps of both groups of elevated ORFs in NMD-deficient strains to those measured in the V81G strains are shown. The arrows mark the number of overlapping transcripts equally present in two groups of accumulating transcripts. Of 415 significantly up-regulated transcripts in V81G 59% (243) were also up-regulated in the upf123Δ, and 50% (208)/47% (194) were up-regulated in the dcp1Δ/xrn1Δ yeast strains. C, the three groups of decreasing transcripts do not show significant overlaps.
lated (see supplemental Table S1). For this reason we wanted to know whether this caused any effect on the telomere metabolism in V81G. Hence the length of the telomeres was compared in the mutant and wild-type strains.

After digestion of genomic DNA isolated from the relevant strains that were incubated at 25 and 37 °C, respectively, a terminal telomere fragment of about 800 bp was released from the bigger telomeric regions (Fig. 8). The band contained Y′ telomere-associated sequences that can be probed by Southern blotting using a DNA fragment that included the telomeric T(G1–3/C1–3)A repeats. The bigger fragments seen on the blot internally contained other subtelomeric fragments that hybridize to the T(G1–3/C1–3)A probe.

As shown previously by other groups (46, 51, 52) the disruption of one or more UPF genes causes a reduction of the average telomere length by about 65 bp. This result was reproduced using genomic DNA of a upf1Δ strain (Fig. 8, lane 3). A complementing UPF1 plasmid was capable of restoring the shortening in the upf1Δ strain. V81G mutant strains cultured at 25 °C developed a shortening of telomeres in the same range as the upf1Δ strains dependent on their population doublings (PDs) (Fig. 8, lanes 4–8). Newly isolated V81G strains from 5-fluoroorotic acid screening showed the same telomere length as wild-type strains of the same age tested in parallel. Shorter telomeres were observed when the PD number of the mutant strains exceeded 25. The connection between the strength of telomeric erosion and the age of the mutant is also supported by the observation of decelerated growth with increasing PDs of a cultured strain (data not shown). Interestingly hardly any telomeric signals were obtained when probing V81G genomic DNA samples that were isolated from cultures incubated at 37 °C for 12 h. This correlates with the observed apoptotic cell death confirmed by TUNEL analysis and caspase activation assays. Taken together, the data suggest that the mutated HYP function caused a short telomere phenotype that is similar to that seen when the NMD pathway is inactive.

**DISCUSSION**

In this work phenotypic effects of a distinct temperature-sensitive point mutated variant of eIF5A were investigated. Valentini et al. (20) provided the first evidence for the importance of the proline residue at position 83 in yeast Hyp2p. This region was further investigated in this study using the human protein. Interestingly the analogous proline 82 substitution resulted in a lethal phenotype further underscoring the importance of this residue within the protein. Mutational analysis of neighboring amino acids gave rise to the useful temperature-sensitive mutant V81G. Homology modeling of the tertiary structure of human eIF5A1 (53) revealed that it consists of two domains similar to those known from hyperthermophilic Archaea (54). By way of analogy residue 81 of the human protein is most likely positioned at the beginning of a flexible hinge region connecting both domains. The glycine mutation therefore may change the orientation of both domains with respect to each other possibly bringing about a disturbance in function.

Because of the pivotal cellular role of eIF5A we were interested in gaining more insight into the function of the human protein. A large library of human eIF5A1 mutants was screened resulting in the discovery of specific temperature-sensitive yeast strains. It is of note that human eIF5A1 permitted normal growth of yeast cells expressing the allele as the unique source of eIF5A (4, 5). This also indicates that the high degree of conservation of eIF5A ensures the same basic functions of the protein in S. cerevisiae and higher eukaryotes.

To analyze phenotypic effects it was important to test the viability of growth-arrested V81G cells. As
eIF5A Yeast Mutant Accumulates NMD Transcripts

shown in Fig. 3 a significant reduction was shown after 6 h at 37 °C. At the same time the mutant showed initial signs of DNA fragmentation and activation of caspases, which are diagnostic markers of apoptotic death (35, 55). Other researchers have also demonstrated the involvement of eIF5A in the apoptotic processes (56, 57). The induction of apoptosis is often linked to the control of the cell cycle and the DNA damage checkpoint. Several indications exist that eIF5A is involved in cell cycle progression (56, 57). The number of overlapping ORFs with the core transcripts of the upf1Δ strains was about 10% higher than those of xrn1Δ and dcp1Δ strains (Fig. 6). However, further experiments will be necessary to clarify the exact role of eIF5A within the pathway. At least one-third of the accumulating transcripts from the V81G strain were not found among those of NMD-deficient strains. The hypusine-containing protein has been found to be essential in yeast, whereas the knock-out of NMD does not lead to a cell cycle arrest and apoptotic death. These facts are strongly suggestive of the involvement of eIF5A in further biological processes.

The accumulation of nonsense transcripts and the stabilization of the CYH2 pre-mRNA were already detected at the permissive temperature. A short incubation time of 60 min at 37 °C was sufficient to increase the CYH2 half-life by a factor of 15. These facts suggest that the mRNA degradation deficiency is a direct rather than an indirect effect. Measurement of mRNA steady states of selected transcripts confirmed the results obtained by the HDOA analysis (Fig. 7). Evidence that these elevated mRNA levels in the mutant were related to a defect in the Upf-dependent mRNA decay pathway was supplied by the determination of the mRNA half-lives of three NMD-dependent transcripts. The TDH2 and RPS5 mRNAs were not found among the group of elevated RNAs, neither in the NMD-deficient strains nor in the V81G strain, and did not show delayed decay. These findings contradict the interpretation that a general defect in mRNA degradation is present in the mutant.
V81G strains displayed a distinct impairment of the telomere positioning effect. In addition the microarray analysis also revealed the accumulation of transcripts of genes playing a direct role in the metabolism of telomeres and of the catalytic subunit of the telomerase itself (supplemental Table S1). We also showed that the V81G mutation leads to a short telomere phenotype (Fig. 8). The same observations were described for NMD-deficient yeast strains that also develop a shortening of telomeres in the range seen in V81G strains.

There is solid evidence that the NMD pathway is essential for telomere metabolism and telomerase function in yeast (51, 52, 46) and that several genes that control telomerase activity like STN1, EST3, and EST1 are regulated by NMD as well (51). The EST1 and EST2 transcripts were found to accumulate, and the EST1 mRNA was found to be stabilized in V81G cells. Additionally it is known that increased levels of telomerase-regulating genes cause short telomere phenotypes, and overexpression of STN1 and EST2 leads to reduced telomeric silencing (51) similar to that seen in V81G. Therefore, the results strongly suggest that the telomeric effects caused by the eIF5A mutation could be explained via the influences of eIF5A on the NMD pathway, disturbing the natural expression levels of telomerase-regulating genes. Another possibility that is not mutually exclusive to this would be that the stabilization of special transcripts could lead to a down-regulation of translation of their corresponding proteins causing a lack of function of these genes and leading to a dysfunctional telomerase or reduced telomeric silencing.

As the mutant form of eIF5A was expressed at a level comparable to the wild type at 25 °C (Fig. 2) the telomeric effects cannot be due to a decreasing eIF5A protein amount but point to further effects caused by the mutant already at the permissive temperature. Thus, the results gained from V81G at this temperature are relevant even if they do not obviously contribute to its strong temperature sensitivity.

There is evidence for eIF5A involvement in cellular senescence (21, 22, 23). We observed that the growth of the V81G mutant cells significantly decelerated with increasing population doublings indicative of a senescent phenotype. Yeast strains deficient in expressing the telomerase cofactors EST1 and EST3, which are both substrates of NMD, also show characteristics of accelerated aging (59). This points to the involvement of eIF5A in the senescence processes that could be explained by its NMD-dependent regulatory effect on telomere maintenance.

In all Archaea investigated so far deoxybypusine synthase and eIF5A have been found to be highly conserved proteins indicating a related function for both proteins in Archaea and eukaryotes. Upf1p is a highly conserved RNA helicase in the NMD pathway, and it is highly likely that it has evolved from an ancient ancestor of Archaea and eukaryotes (60), and RNA metabolism itself is conserved in all eukaryotic systems (61). This points to a co-evolution of eIF5A and NMD proteins and suggests a functional connection between these factors.

The observed partial reduction of translation when depleting eIF5A in yeast (8, 9) raised the hypothesis that eIF5A might have a role in the translation/degradation of a specific subset of mRNAs. Further work is needed to prove whether this subset overlaps with the group of genes regulated by NMD. eIF5A would therefore influence the stability of a section of these NMD-dependent mRNAs before translation. The recognition of nonsense mutations and initiation of translation are intimately linked processes located at ribosomes. It is thus all the more interesting that eIF5A has been found to interact with the ribosomal protein L5 (62). In addition the direct interaction with translating 80 S ribosomes dependent on RNA and the hypusine modification has recently been shown (63).

In summary the eIF5A function is slowly beginning to yield to current molecular biological analysis. In the future elucidating the detailed mechanistic connection of eIF5A and NMD will be of great interest.

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