A Short C-terminal Domain of Yku70p Is Essential for Telomere Maintenance*

Received for publication, March 27, 2000, and in revised form, May 11, 2000
Published, JBC Papers in Press, May 18, 2000, DOI 10.1074/jbc.M002588200

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The Yku heterodimer from Saccharomyces cerevisiae, comprising Yku70p and Yku80p, is involved in the maintenance of a normal telomeric DNA end structure and is an essential component of nonhomologous end joining (NHEJ). To investigate the role of the Yku70p subunit in these two different pathways, we generated C-terminal deletions of the Yku70 protein and examined their ability to complement the phenotypes of a yku70Δ strain. Deleting only the 30 C-terminal amino acids of Yku70p abolishes Yku DNA binding activity and causes a yku70Δ mutant phenotype; telomeres are shortened, and NHEJ is impaired. Using conditions in which at least as much mutant protein as full-length protein is normally detectable in cell extracts, deleting only 25 C-terminal amino acids of Yku70p results in no measurable effect on DNA binding of the Yku protein, and the cells are fully proficient for NHEJ. Nevertheless, these cells display considerably shortened telomeres, and significant amounts of single-stranded overhangs of the telomeric guanosine-rich strands are observed. Co-overexpression of this protein with Yku80p could rescue some but not all of the telomere-related phenotypes. Therefore, the C-terminal domain in Yku70p defines at least one domain that is especially involved in telomere maintenance but not in NHEJ.

While DNA double-stranded breaks are repaired mainly by nonhomologous end joining (NHEJ) in higher eukaryotes, in the yeast Saccharomyces cerevisiae double-stranded breaks are predominantly repaired by homologous recombination (6). Therefore, factors involved in NHEJ in S. cerevisiae eluded detection for a long time. 6 years ago, we identified a DNA-binding protein in yeast sharing significant functional and sequence homology with the human Ku heterodimer. This protein complex, Yku (formerly known as Hdp1), is a heterodimer composed of a subunit of 70 kDa, Yku70p (formerly Hdp1p), and one of 80 kDa, Yku80p (formerly Hdp2p) (7, 8).

Yku binds specifically to DNA ends in a sequence-independent manner (7). Like human Ku, the Yku heterodimer is involved in DNA repair processes. Recently, it has been shown that the Yku protein is an essential part of the NHEJ pathway in S. cerevisiae (9–11). Yeast cells expressing a functional Yku heterodimer can precisely join cohesive ends of a transformed linearized plasmid, but cells deficient for one Yku subunit display reduced recircularization efficiency. Additionally, in Yku− cells, plasmids are not repaired accurately but in an error-prone way, yielding molecules that underwent losses of up to several hundred base pairs at the joining site (9–11). Several other components of the yeast NHEJ pathway have been identified. Lig4p, a newly discovered yeast DNA ligase is essential for end joining and displays significant amino acid sequence homology with human DNA ligase IV (12). Moreover, Rad50p, Mre11p, and Xrs2p have been identified as important components of NHEJ (13).

The human Ku heterodimer, composed of two subunits of 70 and 86 kDa, binds specifically to dsDNA ends and DNA discontinuities such as gaps and nicks (1, 2). Human Ku together with a 465-kDa catalytic subunit forms the DNA-dependent protein kinase (3). Mutations of any of these three DNA-dependent protein kinase subunits cause severe sensitivity to ionizing irradiation and a defect in V(D)J recombination, indicating a role in DNA double-stranded break repair and recombinalion events in higher eukaryotes (4, 5).

* This work was supported by Deutsche Forschungsgemeinschaft Grant Wi 319/11-3, Project 7, and by Canadian Medical Research Council Grant MT12816 (to R.J.W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Deletion of Yku also affects the transcriptional silencing of genes in close proximity to telomeres, a process called telomere position effect (22). Telomere position effect is severely diminished in Yku− cells, although the repression of the silent mating type loci is maintained normally in the same cells (13, 18, 21). Finally, mutations of the Yku heterodimer affect the subcellular organization of yeast telomeres. In Yku mutant cells, the clustered distribution of telomeric foci is abolished, and telomeric signals are detected in a more dispersed localization throughout the whole nucleus (23).

We were thus interested to know whether there are separate domains that would be implicated specifically in different functions of the Yku heterodimer. Stepwise increased deletions of C-terminal amino acids of Yku70p were generated and expressed in a yeast strain carrying a deletion of YKU70. Taking care that at least the same amount of the truncated protein was expressed as normally wild-type protein, we tested whether these proteins could complement the characteristic phenotypes of the strains lacking Yku. We find that the extreme C terminus of the Yku70p subunit is important for DNA binding, telomere maintenance, and NHEJ. Moreover, the data suggest that the last 25 C-terminal amino acids form a small domain essential for telomere stability but not for DNA binding and NHEJ.

MATERIALS AND METHODS

S. cerevisiae Strains, Media, Growth Conditions, and Transformation—Strains used in this study are W303-1A, W303-1D, and W303-Lyh2 as described elsewhere (7, 8). Yeast cell culturing was performed as described elsewhere (8). Yeast transformation was performed by the lithium acetate method (31).

Gel Retardation Assay—Gel retardation assays were performed as described elsewhere (7).

Construction of Mutated YKU70 Genes—C-terminal truncations were generated by Bal31 digestion of an EcoRI-linearized plasmid pGEM4ZHD1F. The deleted fragments were subcloned into pGEM4Z containing a stop codon in all reading frames and sequenced. This cloning strategy resulted in the addition of several new amino acids to the truncated cDNA. To YKU70-c09, YKU70-c20, and YKU70-c25 the amino acid sequence GYRALDIN has been added, and GNQL has been added to YKU70-c30. A PvuII/EcoRI fragment containing the truncated C-terminal part of the YKU70 gene, and a XhoI/PstI fragment isolated from the plasmid pRS316 containing the promoter region and the N-terminal part of the YKU70 gene were cloned into the plasmid pRS316 (25). For overexpression experiments, the wild-type or mutated YKU70 genes were cloned under control of the ADH1 promoter into the plasmid pA-U (URA3 marker) (8). The wild-type YKU70 gene was cloned under control of the ADH1 promoter into the plasmid pAH (HIS3 marker) (8).

End-joining Assay—To generate the plasmids pRS313-kan and pRS314-kan used for end-joining assays, we isolated the KanMX gene from the plasmid pUG6-32 (32) and cloned it into the multiple cloning site of the plasmid pRS313 and pRS314, respectively (25). Plasmids pRS313-kan and pRS314-kan were digested with the restriction enzyme NcoI to completion as determined by gel electrophoresis. The restriction enzyme was inactivated by treatment at 65 °C for 20 min. End-joining assays were performed as described elsewhere (9). Cells were plated onto SD medium lacking histidine or tryptophan and containing Geneticin, G418, (300 μg/ml) for selection of accurately repaired plasmids. The averages from three independent experiments are given.

RESULTS

Generation of YKU70 Deletion Mutants—The Yku heterodimer binds to dsDNA ends with high affinity. Gene disruption of either subunit results in loss of Yku DNA end binding activity in crude extracts (7). To generate C-terminally truncated Yku70 proteins, we digested the coding sequence beginning at the C′-end, using Bal31 nuclease (see “Materials and Methods”). This procedure deleted 30–235 amino acids from the C terminus. We then expressed the truncated proteins under control of the YKU70 promoter from a yeast single copy plasmid in a yku70 deletion strain and measured DNA end binding activity in crude extracts.

In this first set of experiments, we could not obtain truncated Yku70 proteins that completely lacked Yku end binding activity (see below; Fig. 3). Therefore, the C-terminal 30 amino acids of Yku70p seem to be essential for function of the Yku heterodimer (see data below). To further characterize this small C-terminal domain of the Yku70 protein, we generated three additional mutants (see “Materials and Methods”), removing nine (YKU70-c09p), 20 (YKU70-c20p), and 25 (YKU70-c25p), amino acids from the C terminus (Fig. 1).
Yku70-c09p, Yku70-c20p, and Yku70-c25p are slightly reduced as compared with the overexpressed full-length protein, but protein levels are still significantly higher as when they are expressed from the wild-type promoter (Fig. 2B, lanes 3–5 and 7). Yku70-c30p is detectable under these overexpression conditions, the amount of protein being comparable with the amount of wild-type Yku70p expressed from its own promoter (Fig. 2B, lanes 6 and 7).

In addition, we co-overexpressed both subunits, Yku80p and the truncated Yku70p proteins, respectively, under control of the ADH1 promoter in a yku70Δ/yku80Δ double mutant strain. Analysis of protein extracts by Western blot using the anti-Yku70p-specific antibody indicates that in this situation, the level of full-length Yku70 protein now is at least 20-fold higher than when expressed alone from its own promoter, indicating a stabilization of the Yku70p subunit by the Yku80p subunit (Fig. 2C, lanes 1 and 3). Yku70-c09p, Yku70-c20p, and Yku70-c25p also are overexpressed at levels that are very comparable with the full-length protein (Fig. 2C, lanes 4–6). However, even in these conditions, Yku70-c30p expression is drastically reduced when compared with the other proteins, but the expression level still is significantly higher than Yku70p expression from its own promoter (Fig. 2C, compare lanes 1 and 7).

Deletion of 30 C-terminal Amino Acids Abolishes Yku7p DNA Binding Activity—We then tested mutated versions of the Yku7p protein for their ability to restore the Yku heterodimer DNA end binding activity in a yku7Δ deletion strain.

Truncation of up to 25 C-terminal amino acids of Yku7p appears not to affect DNA binding of the Yku heterodimer. We found no reduction in DNA end binding activities in crude extracts of a yku70 mutant strain expressing Yku70-c09p or Yku70-c20p as compared with expressing the full-length protein (Fig. 3A, lanes 2–6). Although there is a significant reduction in Yku70-c25p protein amounts in these cells (see Fig. 2A), DNA end binding activity as measured in this assay is still very comparable with expressing the wild-type protein (Fig. 3A, lane 7). However, we cannot detect any corresponding Yku-DNA complex after expression of Yku70-c30p (Fig. 3A, lane 8). From these results, we conclude that the C-terminal 25 amino acids of Yku7p are not essential for DNA binding. Since the DNA binding activity depends completely upon the formation of the Yku heterodimer (7, 8), these results also demonstrate that these amino acids are expendable for formation of the heterodimer.

To verify these results, we measured Yku DNA binding activity in strains overexpressing the truncated Yku70p versions under the ADH1 promoter. We did not detect any changes in DNA binding activity of all C-terminal truncated Yku70p proteins compared with the expression under control of the YKU70 promoter (data not shown). Although Yku70-c30p is detectable by Western blot analysis in these conditions (Fig. 2B, lane 6), again no DNA binding activity corresponding to the Yku70-c30p/Yku80p heterodimer was observed for this protein (data not shown).

Furthermore, we co-overexpressed both subunits, Yku80p and the truncated Yku70p proteins, respectively, under control of the ADH1 promoter in a yku70Δ/yku80Δ double mutant strain. Using the full-length Yku70p in such experiments yielded a Yku DNA binding activity that is increased at least 20-fold over expressing Yku70p alone under its native promoter (Fig. 3B, lanes 2–4). Overexpression of Yku70-c09p, Yku70-c20p, and Yku70-c25p, respectively, in this setup resulted in no qualitative changes of DNA binding activity as compared with expression under control of the YKU70 promoter (Fig. 3A). However, the DNA binding activity of the active mutant proteins was increased to a level comparable with the overexpressed wild-
A Small and Essential Yku70p C-terminal Domain

Fig. 3. Ykup DNA end-binding assay in yku70 deletion mutants expressing truncated Yku70 proteins. A 39-base pair dsDNA oligonucleotide, PGK12, was labeled with Klenow polymerase and used as a probe for DNA end binding activity. 25 fmol of the oligonucleotide were incubated with crude extract. A, expression under control of the native YKU70 promoter. 30 μg of crude extract prepared from W303–1A (wild-type) or W303aL (yku70) cells expressing the indicated truncated Yku70 proteins under the control of the YKU70 promoter were used. B, simultaneous overexpression of Yku80p and various Yku70 proteins. 5 μg of crude extract prepared from W303aLh2 (yku70/yku80) cells expressing Yku80p and the indicated truncated Yku70 proteins under the control of the ADH1 promoter were used. 5 μg (lane 2) and 50 μg (lane 3) of crude extract prepared from W303–1A (wild type) were used as control.

type protein (Fig. 3B, lanes 4–8). Even in these co-overexpression experiments, Yku70-c30p displayed no DNA binding activity (Fig. 3B, lane 9), although significant amounts of the protein were expressed (Fig. 2C, lane 7). These co-overexpression experiments of the truncated proteins with Yku80p thus confirm the results obtained by expressing them under the control of the native YKU70 promoter.

Telomeres Shorten Gradually with Truncation of the Yku70p C Terminus—Telomeric repeats are dramatically shortened in strains deficient for the Yku heterodimer (11, 17). To investigate the effect of C-terminal deletions of Yku70p on the lengths of telomeric repeats, we tested the effects of an expression of these mutant proteins under control of the YKU70 promoter or when they are overexpressed on telomere length. Yeast telomeres end in about 300 base pairs of a heterogeneous sequence that can be abbreviated C1–3A/TG1–3. Most telomeres that are of larger sizes, depending of where the next internal XhoI site is located (Fig. 4A, lane 2, bracketed).

Expressing Yku70-c09p in yku70 cells under its own promoter yielded slightly shortened telomeric repeat tracts (Fig. 4A, lane 3), although protein levels appear not to be affected in this experiment (Fig. 2A). This shortening, however, is not as severe as observed in a yku70 deletion strain transformed with a vector control (Fig. 4A, lane 1). In strains expressing Yku70-c20p and Yku70-c25p, respectively, the shortening is increased in a stepwise fashion (Fig. 4A, lanes 4 and 5), and the telomeric repeat tracts are about as short as in the deletion strain, when Yku70-c30p is expressed (Fig. 4A, lane 6).

When the various mutant proteins were overexpressed from the ADH1 promoter (Fig. 5B), a slight decrease in telomere length could be detected, and again, overexpression of Yku70-c30p resulted in telomere lengths comparable with a yku70 strain (data not shown). However, this pattern did change in cells in which both one of the various Yku70p constructs and Yku80p were simultaneously overexpressed (Fig. 4B). In this case, no telomere shortening is detectable in a strain expressing either Yku70-c09p, Yku70-c20p, or Yku70-c25p (Fig. 4B, lanes 3–5). In contrast, overexpression of Yku70-c30p together with Yku80p cannot prevent telomere shortening even at these protein levels that are higher than those found for Yku70p in wild-type cells (Fig. 4B, lane 6).

We therefore conclude that the C-terminal amino acids of Yku70p are important for telomere length maintenance and that the DNA binding activity of the Yku heterodimer seems to be essential for telomere length control.

C-terminal Deletion Mutants of Yku70p Exhibit ssDNA Overhang at the Telomeres—It has been shown that yeast chromosome ends acquire a transient, single-stranded overhang of the G-rich strand (G-tails) specifically during S phase (19, 20). In Yku cells, this ssDNA overhang is present throughout the entire cell cycle (18). To investigate whether this increase of telomeric G-tails can be observed in cells expressing the C-terminal truncated Yku70 proteins, we analyzed the telomere structure in such cells by in-gel hybridization to native DNA.
For Yku70-c09p, we observed a slight increase of detection of control of the ADH1 yku70 W303aL cells (A overexpressing truncated Yku70 proteins. DNA loaded in labeled 1-kilobase pair ladder DNA. Note that there is significantly less lanes 11 signals were detectable (Fig. 6 yku70 mutants expressed under control of the To again avoid influences of reduced protein levels, we used the Xho ligation, DNA was separated by gel electrophoresis and analyzed by nondenaturing in-gel hybridization using a 22-mer C1–3A oligonucleotide as a probe. Lane 1, control ssDNA; lane 2, control dsDNA; lanes 3 and 4, pAU vector control; lanes 5 and 6, pAU-YKU70; lanes 7 and 8, pAU-YKU70-c09; lanes 9 and 10, pAU-YKU70-c20; lanes 11 and 12, pAU-YKU70-c25; lane 13, marker, end-labeled 1-kilobase pair ladder DNA. Note that there is significantly less DNA loaded in lane 9 as compared with the other lanes (see B); thus, the signal for ssDNA in this lane is underrepresented. B, the same gel as in A after denaturation of the DNA in the gel and rehybridization to the same probe.

To again avoid influences of reduced protein levels, we used the yku70 mutants expressed under control of the ADH1 promoter. For Yku70-c09p, we observed a slight increase of detection of G-tails as compared with the wild type (Fig. 5, lanes 5–8). Yku70-c20p- and Yku70-c25p-expressing cells clearly displayed a significant increase of the G-tail signals, albeit not reaching the strength observed in yku70 cells (Fig. 5, lanes 9–12, 3, and 4).

In cells co-overexpressing Yku80p and Yku70-c09p, no G-tail signals were detectable (Fig. 6A, lanes 5 and 6), while cells overexpressing Yku70-c25p together with Yku80p displayed weak but clearly perceptible G-tail signals (Fig. 6A, lanes 7 and 8). Co-overexpression of Yku70-c30p and Yku80p resulted in G-tail signals nearly as strong as found in yku70 cells (Fig. 6A, lanes 3, 4, 9, and 10). These results confirm our finding that the DNA binding activity of Yku80p, abolished in a Yku-c30p mutant, is essential for maintenance of telomere integrity. Deletion of 9 amino acids from the C terminus has only a minor effect on telomere length and single-strandedness of the chromosome end, and these effects can be suppressed by overexpressing the mutated Yku heterodimer. Deletion of 20 or 25 amino acids from the C terminus results in a significant increase in single-stranded G-tails, and overexpression of Yku70-c25p together with Yku80p does not restore the chromosomal end structure to wild-type level.

The C-terminal Domain of Yku70p Is Not Involved in End Joining— Yeast cells normally repair DNA double-stranded breaks by homologous recombination. In addition, a second pathway exists in yeast, where nonhomologous DNA bearing cohesive ends can be precisely joined without a loss or addition of nucleotides. It has been shown that this NHEJ pathway in S. cerevisiae depends on the presence of a functional Yku heterodimer (9). To investigate the influence of our Yku70p mutations on NHEJ, we generated pRS313-kan and pRS314-kan plasmids in which the KanMX gene was cloned into the CEN/ARS plasmid pRS313 and pRS314, respectively (25). The respective plasmids were linearized using NcoI, a restriction enzyme cutting inside the start codon of the KanMX gene. Yeast cells are not able to replicate such linear plasmids, and recircularization is an essential step for colony formation on selective medium. After transformation of linearized and supercoiled control plasmids into yeast strains expressing the different yku70 mutants under control of the YKU70 promoter, the cells were plated onto His† (pRS313-kan) or Trp† (pRS314-kan) medium containing G418. Cells capable of joining the ends of the linearized plasmids in a precise way can grow on His† / G418 (Trp† /G418) plates. Cells deficient in NHEJ will often lose genetic information at the site of linearization. This results in a nonfunctional KanMX gene, and the cells will be unable to grow on medium containing G418.

Strains expressing Yku70-c09p and Yku70-c20p, respectively, under control of the YKU70 promoter, on average formed the same number of colonies on G418 medium as a wild-type strain in this end-joining assay, indicating that these truncations did not influence the end-joining capability of Yku (Table I). The Yku70-c25p-expressing cells displayed a decrease in accurate end joining to 60% of the wild-type (Table I). However, since Yku70-c25p is present at reduced levels in these cells (Fig. 2A), this reduction in NHEJ could simply reflect a reduced availability of Yku heterodimer for NHEJ. Overexpression of Yku70-c25p from the ADH1 promoter restores the protein levels for this truncation to at least wild-type levels (Fig. 2B), and NHEJ efficiency now is also restored to a wild-type level (Table I). The strain overexpressing Yku70-c30p formed no colonies on G418 and behaved in this assay like a yku70-deficient strain carrying the vector control.

From these data, we conclude that deletion of the C-terminal 25 amino acids from Yku70p does not significantly influence
the end-joining activity of the Yku heterodimer. DNA binding activity, however, appears essential for NHEJ, since our construct that abolishes DNA binding activity, Yku70-c30p, also abolishes end joining.

**DISCUSSION**

The Yku heterodimer is a DNA end-binding protein involved in at least two different pathways: maintenance of a proper chromosomal end structure and NHEJ. Here we used C-terminal truncation mutants of the Yku70 subunit to identify and characterize separate domains involved in these functions.

Our data indicate that DNA binding activity is essential for all functions of the Yku heterodimer. The one mutation, a deletion of 30 C-terminal amino acids in Yku70-c30p, that abolishes DNA binding activity in all conditions tested, causes a Yku70- phenotype; telomeres are very short, displaying G-tails, and NHEJ is not functional. This is not simply due to the instability of the truncated protein, since overexpression of Yku70-c30p from the ADH1 promoter results in protein levels that are comparable with those found for Yku70p in wild-type cells (Fig. 2B).

On the other hand, the extreme C terminus of Yku70p does appear to define at least one domain involved especially in maintenance of the telomere. This conclusion is mainly based on our observations made using cells expressing a version of Yku70p with the C-terminal 25 amino acids deleted (Yku70-c25p). When this protein is overexpressed from a strong promoter, detectable protein levels are at least as high as those observed for the wild-type protein expressed from its native promoter (Fig. 2). Using these conditions, our data show that DNA binding capacity conferred by this protein is indistinguishable from the wild-type protein (Fig. 3), and NHEJ is fully functional (Table I). However, these same cells still display shortened telomeres (Fig. 5B) and clearly detectable ssDNA overhangs of the 3'-ends (Fig. 5A). These data indicate that the extreme C-terminal domain of Yku70p is specifically involved in maintaining telomere integrity, while this domain is dispensable for NHEJ, provided that the protein is expressed at levels comparable with the wild-type protein. Therefore, for the first time, we were able to separate Ykup functions in telomere maintenance from functions related to NHEJ.

Simultaneous overexpression of Yku70-c25p and Yku80p results in a dramatic increase of active protein able to bind to DNA (Fig. 3B). Nevertheless, telomeres of these cells still display weak G-tail signals (Fig. 6A). These results indicate that Yku70-c25p-associated phenotypes cannot simply be complemented by increasing the amount of active protein above the levels found for the wild-type protein.

In extracts derived from Yku70-c30p-expressing cells, DNA end binding activity is completely abolished, while in extracts derived from Yku70-c25p-expressing cells, DNA binding seems not to be affected. Thus, deleting only 5 amino acids more from the Yku70-c25p construct completely abolishes DNA binding of the Yku complex and renders it nonfunctional. A previous study examining the human Ku70 subunit has identified nine domains conserved between human, mouse, Drosophila, and S. cerevisiae, respectively (see Fig. 1) (26). Deletion of the 159 C-terminal amino acids of human Ku70p, including domains 8 and 9, abolished DNA binding activity (26). Another study has shown that deletion of 89 amino acids from the C terminus of human Ku70p, thereby truncating domain 9 by 7 amino acids, does not influence DNA binding activity, but DNA repair capacity of the Ku heterodimer appears to be affected (27). Deletion of 67 amino acids from the C terminus of human Ku70p abolishes DNA binding activity analyzed by gel shift assay but not in an immunoprecipitation assay (28). In the yeast Ku70 protein, the conserved domain 9 is located closer to the C terminus than in human or mouse Ku70p. Therefore, deletion of 30 amino acids truncates domain 9 by 9 amino acids (Yku70-c30p, Fig. 1). This deletion abolishes the DNA binding activity of the Yku heterodimer as measured in our assays, while truncation of domain 9 by 4 amino acids in Yku70–25p does not influence DNA binding activity. These results indicate that this domain 9 is essential for DNA binding of the Yku heterodimer, since only the last 4 amino acids of this domain may be dispensable for DNA binding.

It has been shown that DNA binding activity of Ku depends on the formation of the Ku70-Ku80 heterodimer. For the human protein, heterodimerization seems to be blocked if domains 8 and 9 are deleted (26). Two dimerization regions, amino acids 1–115 and 430–482, respectively, have been identified in human Ku70p (28). Consistent with these results, pull-down assays using human Ku70p deletions fused to glutathione S-transferase have indicated that there is a weak Ku70p-Ku80p interaction domain in the N-terminal part (amino acids 1–136) of human Ku70p and a strong one located in the C-terminal part (amino acids 449–578) (29). In addition, it has been shown that the stability of human Ku subunits not assembled in the heterodimeric complex is clearly reduced (30). While we do not know whether a heterodimerization domain is affected in Yku70-c30p, a lack of heterodimerization might lead to a decreased stability of the protein, as observed for the human Ku. The stabilities of the truncated Yku70 proteins generated in the present study are only slightly reduced for Yku70-c09p and Yku70-c20p, while Yku70-c25p protein levels are significantly reduced when it is expressed from its native promoter, and Yku70-c30p is virtually nondetectable in such an experiment (Fig. 2A). Nevertheless, using co-overexpression of Yku70-c30p with Yku80p, we could achieve Yku70-c30p protein levels that are comparable with Yku70p in wild-type cells (Fig. 2C, compare lanes 1 and 7), although Yku70-c30p levels still are drastically reduced when compared with when Yku70p was co-expressed with Yku80p (Fig. 2C, compare lanes 3 and 7). This instability of Yku70-c30p may thus be an indication for a diminished heterodimerization followed by degradation of the free Yku70-c30 protein. Further experiments are required to determine whether interaction with Yku80p is prevented in the Yku70-c30p mutant, thereby abolishing Yku function, or whether the effect is due to a lack of a combination of both dimerization and DNA binding.

Shorter stepwise deletions of C-terminal amino acids did result in slight, but detectable, effects on telomere structure but did not interfere with NHEJ. Thus, as mentioned above,
the terminal 25 amino acids of Yku70p appear to form a special domain involved only in telomere stability but not in DNA binding or end-joining activity of Ykup. Interestingly, these 25 amino acids contain 8 lysine residues (Fig. 1). Deletion of 3 of these lysine residues in the mutant Yku70-c09p causes a weak decrease in telomere length when compared with wild-type cells. This decrease is more pronounced in Yku70-c20p, where 6 of the 8 lysine residues are deleted. All 8 lysine residues are removed in the Yku70-c25p mutant, and shortening of telomeres is almost as severe as observed in a yku70-deficient strain. Lysine residues may be important for interacting with yet unidentified protein(s). However, since full NHEJ activity is associated with these phenotypes. For instance, while cells expressing Yku70-c25p at levels comparable with the wild-type protein, this lysine-rich domain at the C terminus does not appear to be essential for the end-joining activity of the Yku heterodimer.

Stepwise truncation of the C terminus additionally induces a stepwise increase in temperature sensitivity for growth (data not shown), which appears to roughly correlate with telomere shortening and an increase in ssDNA detectable at the telomeres. However, again, proficiency for NHEJ clearly does not correlate with these phenotypes. For instance, while cells expressing Yku70-c20p display no decrease in NHEJ (Table I), they are detectably temperature-sensitive (data not shown), which appears to roughly correlate with telomere shortening and an increase in ssDNA detectable at the telomeres of the G-rich telomeric strands is detectable (Fig. 5A). These data are consistent with the hypothesis that the temperature-sensitive growth defect observed in Yku− mutants is not associated with a lack of NHEJ but rather is due to defects in telomere maintenance.

Acknowledgments—We thank B. Meier, M. Jurk, H. Ibelgaufts, K. Marbach, and S. Gravel for helpful discussions and critical advice.

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