Ku Is Important for Telomere Maintenance, but Not for Differential Expression of Telomeric VSG Genes, in African Trypanosomes*

Received for publication, January 17, 2002, and in revised form, March 25, 2002
Published, JBC Papers in Press, March 27, 2002, DOI 10.1074/jbc.M200550200

Colin Conway, Richard McCulloch‡, Michael L. Ginger§, Nicholas P. Robinson, Alison Browitt, and J. David Barry¶

From the Wellcome Centre for Molecular Parasitology, University of Glasgow, Anderson College, 56 Dumbarton Road, Glasgow, G11 6NU, Scotland, United Kingdom

Trypanosome antigenic variation, involving differential expression of variant surface glycoprotein (VSG) genes, has a strong association with telomeres and with DNA recombination. All expressed VSGs are telomeric, and differential activation involves recombination into the telomeric environment or silencing/activation of subtelomeric promoters. A number of pathogen contingency gene systems associated with immune evasion involve telomeric loci, which has prompted speculation that chromosome ends provide conditions conducive for the operation of rapid gene switching mechanisms. Ku is a protein associated with eukaryotic telomeres that is directly involved in DNA recombination and in gene silencing. We have tested the hypothesis that Ku in trypanosomes is centrally involved in differential VSG expression. We show, via the generation of null mutants, that trypanosome Ku is closely involved in telomere length maintenance, more so for a transcriptionally active than an inactive telomere, but exhibits no detectable influence on DNA double strand break repair. The absence of KU and the consequent great shortening of telomeres had no detectable influence either on the rate of VSG switching or on the silencing of the telomeric promoters of the VSG subset that is expressed in the tsetse fly.

The heterodimeric protein Ku, which consists of the subunits Ku70 and Ku80 (or Ku86), associates tightly in a sequence-independent fashion with free ends of double strand DNA and has been associated with a range of nuclear functions in different eukaryotes (reviewed in Refs. 1–3), including DNA repair, retrotransposition (4), gene silencing, transcriptional reinitiation (5), chromosome maintenance, and chromosome localization. Recently, homologues possibly associated with DNA repair have been discovered also in prokaryotes (6, 7). Ku is central to the non-homologous end joining (NHEJ)³ pathway for DNA double strand break repair in which double strand breaks with dissimilar ends can be rejoined with minimal error (8). A main function of Ku is to bind to DNA and recruit proteins, such as the catalytic subunit of mammalian DNA-dependent protein kinase, that catalyze steps in the repair process, although a weak helicase activity has been ascribed by some to Ku itself (1). Ku also plays a major role in telomere length maintenance in yeasts such that the deletion of KU genes causes shortening of the telomere tract (9–11). This function of Ku again is thought to operate mainly through its interaction with other molecules (12, 13). The NHEJ function of Ku is prevented from acting at telomeres by other telosome proteins such as Taz1 (14), and indeed the presence of Ku is required for the prevention of recombination at the free ends of telomeres (15, 16).

† A Royal Society University Research Fellow.
‡ A Royal Society University Research Fellow.
§ Present address: School of Biological Sciences, University of Manchester, 2.205 Stopford Building, Oxford Road, Manchester M13 9PT, UK.
¶ A Wellcome Trust Principal Research Fellow. To whom correspondence should be addressed. Tel.: 0044141-330-4875; Fax: 0044141-330-5422; E-mail: j.d.barry@bio.gla.ac.uk.
³ The abbreviations used are: NHEJ, non-homologous end joining; TPE, telomere position effect; VSG, variant surface glycoprotein; BES, bloodstream expression sites; ORF, open reading frame; RT, reverse transcription; MMS, methyl methanesulfonate; PHR, primary homology regions; wt, wild type.

* This work was supported by the Wellcome Trust and the Royal Society. 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.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AJ307890, AJ311945.

The heterodimeric Ku protein, which consists of the subunits Ku70 and Ku80 (or Ku86), associates tightly in a sequence-independent fashion with free ends of double strand DNA and has been associated with a range of nuclear functions in different eukaryotes (reviewed in Refs. 1–3), including DNA repair, retrotransposition (4), gene silencing, transcriptional reinitiation (5), chromosome maintenance, and chromosome localization. Recently, homologues possibly associated with DNA repair have been discovered also in prokaryotes (6, 7). Ku is central to the non-homologous end joining (NHEJ)³ pathway for DNA double strand break repair in which double strand breaks with dissimilar ends can be rejoined with minimal error (8). A main function of Ku is to bind to DNA and recruit proteins, such as the catalytic subunit of mammalian DNA-dependent protein kinase, that catalyze steps in the repair process, although a weak helicase activity has been ascribed by some to Ku itself (1). Ku also plays a major role in telomere length maintenance in yeasts such that the deletion of KU genes causes shortening of the telomere tract (9–11). This function of Ku again is thought to operate mainly through its interaction with other molecules (12, 13). The NHEJ function of Ku is prevented from acting at telomeres by other telosome proteins such as Taz1 (14), and indeed the presence of Ku is required for the prevention of recombination at the free ends of telomeres (15, 16).

† A Royal Society University Research Fellow.
‡ A Royal Society University Research Fellow.
§ Present address: School of Biological Sciences, University of Manchester, 2.205 Stopford Building, Oxford Road, Manchester M13 9PT, UK.
¶ A Wellcome Trust Principal Research Fellow. To whom correspondence should be addressed. Tel.: 0044141-330-4875; Fax: 0044141-330-5422; E-mail: j.d.barry@bio.gla.ac.uk.
³ The abbreviations used are: NHEJ, non-homologous end joining; TPE, telomere position effect; VSG, variant surface glycoprotein; BES, bloodstream expression sites; ORF, open reading frame; RT, reverse transcription; MMS, methyl methanesulfonate; PHR, primary homology regions; wt, wild type.

* This work was supported by the Wellcome Trust and the Royal Society. 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.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AJ307890, AJ311945.
Two constructs were used to delete each gene, using two rounds of transformation. Each construct contained ~400 bp of targeting sequence derived from sequences immediately upstream and downstream of either ORF, meaning that transformants delete the entire ORF.

Targeting flanks for each gene were amplified from genomic DNA using primers flanking the ORF. KU70 RE-EXP FOR/REV and KU80 RE-EXP FOR/REV primers were used to amplify a 1.1 kb and a 1.0 kb targeting flank from the Sanger and TIGR trypanosome genome databases, respectively. The final transformation constructs were digested with NotI and cloned independently into both pTIB and pTP (gifts of M. Cross and P. Borst, the Netherlands Cancer Institute). pTP contains the 400-bp blasticidin S deaminase ORF (BSP) flanked by 240 bp of 5' and 330 bp of 3' processing signals derived from the 5' and 3' flanks of T. brucei actin ORF. pTIB contains identical processing signals flank- ing sequence confirmed the correct integration of the actin intergenic region rather than the 5' ORF sequence. The pCC101 was used to reintroduce the 5' ORF sequence. Each transformant was selected with 10 μg/ml 1-blasticidin or 1 μg/ml puromycin.

Correct integration of cassettes into both KU loci was determined by Southern blot analysis. Genomic DNA from KU wild type and mutant cell lines was digested with EcoRI and separated on a 0.6% agarose gel. The gel was subsequently Southern-blotted onto Hybond XL (Amer- sham Biosciences) and probed with the same KU70 and KU80 ORF-derived sequences used to probe the genomic library. Following hybridization, the blots were washed to a final stringency of 0.2× SSC, 0.1% SDS at 65 °C. A 12-kb fragment corresponding to intact KU70 was detected in both wild type and heterozygous cell lines but was absent in the homozygous mutants. Two bands were present in KU70 wild type lanes due to allelic differences (9 and 15 kb). One of these bands was lost upon disruption of the first allele, whereas both were lost in KU80 homozygous mutants. Probing the blots with KU70 and KU80 5'-flanking sequence confirmed the correct integration of the BSR and PAC resistance cassettes. Again, the blots were washed to a final stringency of 0.2× SSC, 0.1% SDS.

Re-expressing KU70 in the Homozygous Knockouts—Construct pRNC101 was used to reintroduce KU70 in its native location in the homogygous KU70 ORF, along with 620 bp of 5'-flanking sequence, which was isolated as a 2.9-kb PCR-amplified fragment using the following primers: KU70RE-EXP FOR, 5'-GGCCAATTTAACGCTCAGTGTCATGTCC- C-3', KU70RE-EXP REV, 5'-GATCCGTATTACGCCGCGGAGGAGGAAACC-3 (ApaI underlined). The fragment was amplified using a 10-min denaturation step followed by 30 cycles of 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C. This reaction was carried out using Pfu DNA polymerase, and the fragment was cloned into a unique ApaI restriction enzyme site in pNR101. This placed the KU70 sequence upstream of a construct with ~400 bp of actin intergenic sequence at the 5’ flank of the bleomycin resistance gene and 330 bp of an α-tubulin intergenic region at the 3’ end. In the homogygous mutants, the 620 bp of KU70 5’ flank and α-tubulin intergenic region act as targeting flanks, allowing KU70 to be reintroduced into the disrupted locus. The only potential difference in expression is that 3’ processing is provided by the actin intergenic region rather than the natural KU70 3’ signals. The pCC101 was linearized with NotI prior to electroporation, and transformants were selected on semisolid agarose plates containing 2.0 μg/ml bleomycin (Invitrogen). Integration of re-expression cassettes was tested by Southern blot analysis; genomic DNA was digested with EcoRI, separated on a 0.6% agarose gel, Southern-blotted, and probed with the KU70 ORF sequence. Each transformant had integrated the KU70 re-expression cassette, as expected. The control construct used in this analysis (pRM450) contains the bleomycin-resistant gene (BLE) surrounded by the same processing signals as in pTIB/pTF and targets the tubulin array. This was cut with NotI and blunted using Klenow fragment of DNA polymerase. This construct was electroporated into KU70 cells and screened by Southern blots. Transformants were selected with 3 μg/ml bleomycin, and one clone was identified. This was then further characterized by PCR to confirm the correct integration of the 3’ re-expression cassette.
puncture 24 h later, and trypanosomes were isolated and cloned in 96-well plates as described previously (40). The number of switched clones that grew through was used to calculate the frequency of switching events and assumed an in vitro doubling time of 8 h.

Viability and Growth of ku70 Mutants in the Presence of DNA Damaging Agents—To test the ability of ku70 mutants to grow and divide in the presence of the DNA damaging agents methyl methanesulfonate (MMS; Sigma) or phleomycin (Cayla), a subconfluent viability assay was adopted. Cell lines were grown to a maximum density of $2 \times 10^6$ cells/ml and then diluted to a concentration of $1 \times 10^7$ cells/ml. Each mutant was titrated against wild type trypanosomes to determine the sublethal range. To assess the effect of MMS on growth, the diluted cells were spread over a 96-well culture dish (5 cells/well for MMS, 2 cells/well for phleomycin, minimum densities giving reproducible growth data), and the number of wells with growing trypanosome populations were counted after 7–9 days. In vitro growth rates were measured by diluting mid-log bloodstream trypanosomes to a concentration of $1 \times 10^7$ cells/ml and measuring using a hemocytometer.

**Telomere Length Maintenance Assay**—Wild type, heterozygotic, and homozygotic KU70 and KU80 cell lines were subcloned on semisolid agarose HMI-9 plates. Multiple clones for each were then grown to a density of $1 \times 10^4$ cells/ml, genomic DNA was isolated and digested with EcoRI or AgeI depending on the expression site under analysis. EcoRI cuts 3 kb upstream of VSG2221, which is located in the active expression site. AgeI cuts just within the 5′ end of the coding sequence of the V02 gene in the transcriptionally inactive expression site analyzed. Genomic DNA digests were separated on a 0.6% agarose gel, Southern-blotted onto Hybond XL (Amersham Biosciences), and probed with the amino terminus region of either V02 or VSG2221.

**Analysis of Transcriptional Status of Metacyclic VSGs**—Three previously characterized metacyclic VSG loci were used to analyze the transcriptional status of MVSGs in the procyclic stage. Total RNA from KU70 wild type, heterozygotic, and homozygotic mutant cell lines was prepared using TRIzol. cDNA was generated using random hexamers and Superscript II reverse transcriptase (following protocols from Invitrogen). Integrity of cDNA was tested using a control PCR on cDNA to amplify RNA polymerase I mRNA as described previously (41). Three characterized VSG loci, VSG21.22, 1.61, and 1.63, were examined by PCR for the presence of transcripts both proximal to the promoter and around the VSG. PCR was carried out on the cDNAs with -3 pmol of upstream and downstream primers with an initial 10-min denaturation at 95 °C, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. These reactions were carried out using TaqDNA polymerase (Abgene Ltd.) for each locus except the MVSG1.61 promoter proximal region, where Pfu DNA polymerase was used with 2 mM MgSO4 and a lower annealing temperature of 50 °C. The PCR primers were designed as follows: MVSG1.22 promoter proximal, primer 5′ ends were +29 and +214 from the transcription start site defined from metacyclic nascent transcripts, and VSG coding sequence primer 5′ ends were +426 and +666 from the ATG start codon, which is 2934 bp from the transcription start; MVSG1.61 proximal promoter ends were +28 and +287 from transcription start, and coding sequence ends were -5 and +305 from ATG, which is 2783 bp from the transcription start; MVSG1.63 proximal promoter ends were +59 and +230 from transcription start, and coding sequence ends were +621 and +837 from ATG, which is 2988 bp from the transcription start.

**RESULTS**

Trypanosome KU Homologues Are Not Colinear—As neither KU70 nor KU80 has a high degree of interspecies similarity even at the polypeptide level (42), the T. brucei genome sequence databases were blast-searched individually with all known homologues. Although most hits were unique to each query sequence, some were shared by more than one. Detailed searching of databases, using Smith-Waterman algorithms, supported the view that these common hits did indeed correspond to KU homologues. Using PCR-amplified fragments based on these sequences as probes, full-length trypanosome sequences were obtained by the isolation of overlapping genomic clones from our existing genomic library. Sequence analysis revealed complete open reading frames (trypanosomes are almost fully intronless) potentially encoding polypeptides of 81 kDa for the KU70 homologue and 69 kDa for the KU80 homologue. It was possible to align parts of those sequences with the primary homology regions (PHRs) of other KU homologues by Clustal W analysis (Fig. 1). The predicted sizes of the trypanosome KU proteins are the inverse of what occurs in other organisms, and their designation is complicated further by the general relatedness of Ku70 and Ku80 and the dispersion of homologous sequences throughout the proteins. Our belief that the larger protein corresponds to Ku70 relies on the outcome of blastp and PSI-blast searching, which routinely place it closer to Ku70 than Ku80 proteins, and the presence at its carboxyl-terminal of a partial putative DNA binding SAP domain (6, 43) that has the secondary structure potential of other SAP sequences. Trypanosome Ku80 likewise detects mainly Ku80 sequences in blast searching. It does not have a recognizable DNA-dependent protein kinase binding sequence found at the end of vertebrate Ku80 proteins, but neither do the yeast Ku80 proteins.

Homology-based modeling using Jpred (jura.ekb.ac.uk:8888) reinforced the similarity of the trypanosome sequences to known KU proteins of other organisms and also suggested a likely reason for most of the extra length of the trypanosome Ku70. Essentially all α helices and β strands in the crystal structure of the human protein (44) are present in the predicted trypanosome Ku70 structure, except in the region of the ring that encircles DNA. Although in other species there are about 50 residues between the βC and βM strands, the trypanosome has about 120 amino acids. It is intriguing that trypanosome Ku80 has a typical 75 residues in the corresponding region, revealing an asymmetry between the two monomers in the ring region.

**Null Mutants Have Normal Growth**—To analyze the function of Ku, initially looking for general phenotypes described previously in other organisms and then questioning possible specific roles, we generated null mutants. Genomic Southern analysis revealed that both KU70 and KU80 probably are single copy genes (data not shown), an interpretation subsequently confirmed during gene knockouts. Initially, we deleted genes in bloodstream stages of T. brucei S427 grown in vitro. In the 3174.2 strain, which is marked with the HYG and NEO antibiotic resistance genes in its transcriptionally active telomere, we deleted the first KU70 copy using the puromycin resistance cassette (PAC), obtaining the independent clones, 70P3.1(+/-) and 70P4.2(+/-). Both then had the second allele deleted with the blasticidin resistance cassette (BSR), generating the clones 70P3.1B(-/-) and 70P4.2B(-/-). A number of subclones of each of these were derived, enabling the study of events in individual sublines. A similar approach was used for KU80, except that blasticidin resistance was used first, followed by puromycin. This generated the independent heterozygotic clones 80B1.1(+/-) and 80B1.3(+/-), from which were derived the homozygous mutant clones 80B1.1P(-/-) and 80B1.3P(-/-), respectively.

The first phenotype examined was growth. The population doubling time of wild type 3174 trypanosomes (9.35 and 9.75 h in two experiments) compared well with those of the heterozygotic mutants 70P3.1(+/-) (10.25 h), 70P4.2(+/-) (10.25 h), 80B1.1(+/-) (10 h), and 80B1.3(+/-) (9.12 h), as well as those of the null mutants 70P3.1B(-/-) (9.75 h), 70P4.2B(-/-) (10 h), 80B1.1P(-/-) (9 h), and 80B1.3P(-/-) (9.25 h). Ku therefore is not essential for bloodstream trypanosome growth, and its absence causes no detectable increase in population doubling time, indicating that there is neither a general delay in the cell cycle nor chronic death of some of the population.

---

2 M. L. Ginger, P. A. Blundell, and J. D. Barry, unpublished results.
Ku Is Essential for Maintenance of a Transcriptionally Active Telomere—Telomere length maintenance depends on Ku. In *T. brucei*, it is possible to monitor the length of individual telomeres because of the subtelomeric location of individual VSG genes. By digesting DNA with a restriction enzyme with a site upstream, but not downstream, of a VSG and then probing for that gene, a fragment containing the entire telomere tract can be detected. We have studied two distinct VSGs. The 221 gene is in the transcriptionally active telomere in the bloodstream trypanosomes under study. The VO2 gene has one copy in a silent telomere and a second copy that, being within a chromosome, is flanked on both sides by restriction sites. For that gene, a fragment containing the entire telomere tract upstream, but not downstream, of a VSG site can be detected. We have studied two distinct clones 70P3.1 (H11002) and 70P4.2 (H11001) and the homozygous deletion 80B1.1 (H11002). One subclone from each independent homozygous mutant was used for these experiments, the transcriptionally active 221-telomere fragment was a mean 7.2 kb long (range 3–10 kb) in homozygous null mutant subclones (70P3.1B/H11002, 70P4.2B/H11002, and 80B1.1/H11002), whereas a more extensive analysis was carried out for the VO2 gene. By digesting DNA with a restriction enzyme with a site upstream, but not downstream, of a VSG and then probing for that gene, a fragment containing the entire telomere tract can be detected. We have studied two distinct clones 70P3.1B (H11002) and 80B1.3P (H11002). The number of subclones of 70P3.1B (H11002) and 80B1.3P (H11002) and the homozygous deletion 80B1.1P (H11002) were examined. Similarly, for the VO2 gene has one copy in a silent telomere and a second copy that, being within a chromosome, is flanked on both sides by restriction sites. Fragment sizes for the telomere downstream of the transcriptionally active VO2 gene have not been characterized, so we cannot say how much non-telomere sequence is present in the Agel terminal restriction fragments.

To reintroduce wild type Ku into its own locus using a co-transcribed bleomycin (BLE) resistance cassette (pCC101) and, as a control, we targeted BLE to the tubulin locus (pRM450). Four null mutant subclones (70P3.1B—/—, 70P3.1B—/—, 70P4.2B—/—, 70P4.2B—/—; Fig. 2, lanes 19, 13, and 15) were chosen for this experiment. Analysis of the active expression site (once again using EcoRI to release terminal restriction fragments) showed that in each case, re-expressors harbored longer telomere tracts than did the null mutants (Fig. 3). These were grown for an estimated 18 generations during selection for transformants and then 12 more generations in the absence of bleomycin, at which time they were analyzed for telomere tract length. Taking the fragment sizes for all these KO70 experiments, the transcriptionally active 221-telomere fragment was a mean 7.2 kb long (range 3–10 kb) in homozygous mutant trypanosomes and 10.3 kb long (range 9–15 kb) in the re-expressor trypanosomes. This fragment contains about 3 kb of non-telomere tract sequence. 

A Transcriptionally Inactive Telomere Is Less Prone to Length Changes in Ku Null Mutants—We next looked at the effect disrupting Ku had on a transcriptionally inactive telomere (Fig. 4). This was carried out using restriction digestion with AgeI and Southern blot analysis. In this instance, three of the four subclones used in the 221 experiment were analyzed. The VO2 gene has one copy in a silent telomere and a second copy that, being within a chromosome, is flanked on both sides by restriction sites. Fragment sizes for the telomere downstream of VO2, measured on the same DNA samples and therefore identical lineages as for the 221 analysis, are 9.4 kb (9.1–10 kb) in homozygous mutants and 9.8 kb (9.5–10.5 kb) in re-expressors. The telomere immediately downstream of the VO2 gene has not been characterized, so we cannot say how much non-telomere sequence is present in the AgeI terminal restriction fragments.
Ku Null Mutants Do Not Have a Detectable Deficiency in DNA Double Strand Break Repair—The third general phenotype examined was sensitivity to MMS, which causes both single and double strand breaks (45), and to phleomycin (bleomycin family), which causes double strand breaks directly. The same clones as used for the initial growth analysis were grown in the continuous presence of MMS. Standard growth curves revealed a dose-response effect on the growth of wild type trypanosomes over the range 0.0003–0.0005% MMS. The wild type 3174.2 trypanosomes displayed population doubling times of 9.35 h in the absence of MMS, 13 h at 0.0003%, 16.5 h at 0.0004%, and 28 h at 0.0005%. This compared well with those of the heterozygous mutants where population doubling times were 9.56 h in the absence of MMS, 12.4 h at 0.0003%, 16.6 h at 0.0004%, and 27.5 h at 0.0005%. Homozygous mutants displayed similar population doubling times: 9.13 h in the absence of MMS, 11.75 h at 0.0003%, 15 h at 0.0004%, and 25.25 h at 0.0005%. These results represent an average of two independently obtained data sets. Thus, for both heterozygous and homozygous mutants of both KU70 and KU80, the same dose-response effect occurred, indicating no increase in mutagen sensitivity in the mutants. Confirmation of the apparent lack of increased sensitivity in the mutants came from a clonal growth assay in which five trypanosomes were plated in each well of a 96-well plate and the percentage of wells displaying growth after 96 h was scored. This test is more sensitive but also more variable due to trypanosome founder effects. The assay showed, again, no increased sensitivity to MMS or to phleomycin, which we tested at two trypanosomes per well (Fig. 5).

Ku Null Mutants Are Not Impaired in Bloodstream VSG Switching—Having established some of the general phenotypes predicted for KU genes, we next asked whether there is a specific role in the differential expression of VSG genes. In the bloodstream stage of T. brucei S427, switching occurs at a background rate and is achieved by a number of routes, including transcriptional switching between telomeres and gene duplication into telomeres. The marked strain we have used allows most of these activation mechanisms to be detected (40),...
Ku in Trypanosomes

**FIG. 4. Telomere length maintenance of an inactive expression site in Ku70 re-expressors.** Ku70 homoygous mutant clones re-expressing Ku70 are indicated by */−/+*, whereas clones deleted by */−/−* were transformed with the control plasmid pRM450. Schematics to the right of the panel depict the VO2 VSG loci under investigation. The upper schematic shows the telomeric VO2 expression site with telomere repeats depicted as arrows, VSGVO2 gene depicted as a gray box, and 70-bp repeats depicted as a black box. An AgeI restriction site present upstream of the telomeric VO2 gene is shown, and the probe used for hybridization is depicted. The lower schematic shows the chromosomal internal VO2 gene and national flanking AgeI sites. Genomic DNA from each transformed Ku70 subclone was digested with AgeI, Southern blotted, and probed for VSGVO2. Molecular size markers are indicated to the left of the panel.

providing a convenient means of testing for alteration in switch rate. This assay was applied to the Ku70 mutant lines 70P3.1(+/−), 70P4.2(+/−), 70P3.1B(−/−), and 70P4.2B(−/−). All gave the rate of 0.1–0.6 × 10⁻⁶ switch/cell/generation (Fig. 6), which is typical of wild type cells, there being no evidence for a significant decrease in switch rate in the absence of Ku70.

**FIG. 5. Viability of KU70 mutants in the presence of mutagens.** 3174.2 bloodstream form trypanosomes that had been unaltered in their KU70 gene (wt), had one allele disrupted (KU70+/−), or had both alleles disrupted (KU70−/−) were plated in culture dishes containing increasing concentrations of MMS. The same heterozygous and homozygous mutant lines were tested also in phleomycin, as shown in the lower panel. Surviving populations were counted after 6–9 days and are expressed as a percentage of the total number of wells. The data are means of four readings (duplicate growth experiments of two independent lines). Error bars represent standard deviations.

**FIG. 6. VSG switching frequencies in KU70 mutants.** Wild type cells (3174.2 wt) were compared with two KU70 heterozygous mutants (70P3.1+/−, 70P4.2+/−) and two homozygous mutants (70P3.1−/−, 70P4.2B−/−). Each bar in this graph represents an independent experiment.

Ku Is Not Essential for Silencing of the Naturally Telomeric MVSG Genes—VSG genes are first expressed in the metacyclic stage of the trypanosome life cycle, in the tsetse. Differential expression of the subset of telomeric genes used there, known as MVSG genes, occurs by transcriptional activation. In most life cycle stages, MVSGs are not expressed, but individual ones are activated in individual metacyclic trypanosomes. Here, we tested the hypothesis that the silencing of MVSGs in the procyclic (tsetse midgut) stage is exerted by a Ku-mediated telomere position effect. We have tried repeatedly to transmit T. brucei S427 through tsetse, to no avail (data not shown), so we have been unable to identify MVSGs in this strain. Therefore, we instead used T. brucei EATRO 795, which routinely develops to the metacyclic stage, permitting characterization of the MVSGs encoding the ILTat 1.22, 1.61, and 1.63 VSGs. We deleted both KU70 alleles in procyclic stage T. brucei EATRO 795 grown in vitro, creating the independent heterozygous, puromycin-resistant knockout clones 70+/− and 70+/−2 and then their homozygous, blasticidin-resistant doubly deleted clonal descendants called, respectively, 70−/−1.1 and 70−/−2.1. RT-PCR was used to search for transcripts from
two regions of each MVSG locus. The first, downstream of the transcription start sites (Fig, 7), would detect primary transcripts and stable RNA. The scant availability of metacyclic stage RNA prevented its use as a positive control, so instead of metacyclic cDNA, we used the corresponding trypanosome genomic DNA. Although this control (Fig, 7, lanes 9 and 10, and lanes 5 and 6) are from 70/-1.1, lanes 7 (RT+) and 8 (RT-) are from 70/-1.1 (6), lanes 9 and 10 are from 70/-1.2 (1), and lane 11 is a control PCR using the respective primers on EATRO 795 procyclic genomic DNA. C, RT-PCR amplification of the RNA polymerase I large subunit as a control for cDNA integrity. Lane order is identical to panels A and B, although no genomic DNA control was undertaken. Pol I, polymerase I.

FIG. 7. Transcriptional status of telomeric MVSGs in procyclic form KU70 mutants. RT-PCR analysis was undertaken with RNA from wild type and mutant lines. The telomeric environment of the three MVSGs (1.22, 1.61, and 1.63) examined is depicted in the top diagram; the promoter is shown as a black flag, the small number of 70-bp repeats is shown as a white box, and the VSG gene is shown as a white arrow. Converging arrows below the expression site show the positions of oligonucleotides used for PCR. A, PCR of the cDNA and mock cDNA for each MVSG proximal to the promoter. B, PCR of the three MVSG sequences. Lanes 1 (RT+) and 2 (RT-) shown are for wild type cDNAs, lanes 3 (RT+) and 4 (RT-) are from 70/-1.1, lanes 5 (RT+) and 6 (RT-) are from 70/-1.2, lanes 7 (RT+) and 8 (RT-) are from 70/-1.1 (6), lanes 9 and 10 are from 70/-1.2 (1), and lane 11 is a control PCR using the respective primers on EATRO 795 procyclic genomic DNA. C, RT-PCR amplification of the RNA polymerase I large subunit as a control for cDNA integrity. Lane order is identical to panels A and B, although no genomic DNA control was undertaken. Pol I, polymerase I.

DISCUSSION

We have cloned and characterized, by gene deletion, the trypanosome homologues of Ku70 and Ku80. Our analysis of telomere lengths revealed the telomere shortening phenotype that occurs in other eukaryotes, but the classical role in DNA damage repair by NHEJ was not detected. Our hypothesis that Ku is important in VSG recombinational or transcriptional control was not supported, revealing at least that the Ku-mediated TPE described for S. cerevisiae is not necessary for regulation of antigenic variation.

Due to the limitation that low sequence homology between Ku proteins from different species prevents construction of homology models based on the human Ku structure (44), our identification of these trypanosome sequences has relied on the five primary homology regions (42) and on our observation that most of the determined human α helices and β strands have counterparts, with corresponding spacing, in the trypanosome sequences. In this way, it became apparent that trypanosome Ku70 has an extra, ~70 residues in the ring that encircles DNA, immediately upstream of PHR3. This correlates well with the observed gaps between PHR2 and PHR3: 250 amino acids in human Ku70 (42) as compared with 315 residues in trypanosome Ku70. Although the human Ku80 PHR2-PHR3 gap (274 residues) is collinear with Ku70, that of trypanosome Ku80 is considerably shorter (273 residues) than in its presumed heterodimeric partner; perhaps trypanosome Ku70 has an extra loop available for interaction with other proteins. In common with other simple eukaryotes, trypanosome Ku80 lacks the carboxyl-terminal motif of higher eukaryotes for binding to the DNA-PK catalytic subunit (42), and we cannot find evidence for the catalytic subunit sequence in the (incomplete) trypanosome genome database. The trypanosome Ku70 contains what might be a SAP domain, in common with Ku70 from other species (6, 43).

Our data show that Ku is central to telomere length maintenance in trypanosomes and reveal that, in the presence of Ku, there are marked differences between short and long telomeres and between the transcriptionally active and inactive telomeres examined in the kinetics of telomere lengthening. Although quantification of the extent of telomere loss was complicated by the large terminal restriction fragment sizes involved, regrowth of short, more accurately sized telomeres could be monitored following reintroduction of KU70. The EcoRI terminal fragments liberated from the active telomeric 221 locus in null mutants were, on average, 3.1 kb shorter than in the Ku70 re-expressors after 30 population doublings. This difference was substantially lower for the transcriptionally inactive VO2 telomere, where reintroduction of Ku70 caused an average increase of only 400 bp. transcriptionally active trypanosome telomeres have previously been demonstrated to grow faster than inactive telomeres (46). There was also a growth difference between particularly short (~1 kb) and much longer (>7 kb) telomeres upon re-expression of Ku70; the short telomeres extended by ~170 bp/population doubling, as compared with 103 bp/population doubling for the longer telomeres. Such rapid expansion resembles the growth of newly formed trypanosome telomeres (47). Our findings presumably reflect that telomere capping is less pronounced in transcriptionally active, or new, telomeres. Despite the great decrease in telomere length, no trypanosome clone analyzed appeared to have lost all telomeric sequence, and null mutant clones with terminal restriction fragments as short as 4 kb multiplied at the same rate as wild type cells even after 150 generations (data not shown). A length equilibrium was reached within such periods, indicating that the trypanosome has at least one alternative pathway for telomere maintenance, as has been observed in other organisms (reviewed in Ref. 48).

The main DNA repair role for Ku is in NHEJ reactions (49, 50). We found no difference between wild type, heterozygotic, or homozygotic KU mutants in their sensitivity to MMS, in contrast to rad51 mutants, which display increased MMS sensitivity in the same range of drug concentrations (34). We see also that there is not an increased sensitivity to phleomycin. It appears, therefore, that if Ku plays a role in the repair of trypanosome DNA damage, it would be masked by the greater prevalence of homologous recombination. To some extent, this is what happens in yeast KU mutants, where no increased MMS sensitivity is detectable in diploids, and the background role played by Ku is seen only when the more central homol-
gous recombination gene RAD52 is also inactivated (9, 11, 18, 51), although there is a significant increase in sensitivity to bleomycin in both haploid and diploid stages (52). Indeed, wild type trypanosomes and *S. cerevisiae* nearly exclusively use homologous recombination when transfected, linearized DNA is integrated into the genome. Nevertheless, the trypanosome appears to have more capacity for alternative repair, such as is seen in the persistent resistance to bleomycin, and there is evidence for at least one Rad51-independent homologous recombination pathway (34). We have been unable to find NHEJ in wild type trypanosomes using an assay for recircularization of electroporated, restriction-digested plasmids, although chromosomal integration of linear DNA constructs with non-homologous flanking sequence gives some success. It remains possible that Ku-dependent NHEJ exists in trypanosomes but is not observed in these transformation experiments because DNA integration operates at only the S to M cell cycle phase, where homologous recombination may dominate in higher eukaryotes (53). Perhaps NHEJ is limited to the G1 phase or to non-dividing trypanosome life cycle stages, when limited DNA replication occurs.

There has been much speculation as to why parasite contingency genes are found at telomeres. Besides the trypanosome VSGs, which are transcribed only from telomeric loci in the distinct metacyclic and bloodstream populations and have a large subset of silent genes at minichromosomal telomeres, there are examples in protozoa (25, 54), the fungus *Pneumocystis* (26), and even on linear plasmids in the bacterium *Borrelia hermsii* (55). These systems rely on the existence of a wide set of silent genes (or segments) and the expression of only one gene at a time. There is a constant pressure for expansion of the silent information, which probably is best served by DNA recombination, generating novel sequence combinations. Telomeres may enhance this process as they have high recombination rates and are able to recombine with each other promiscuously, independent of homology elsewhere on the chromosome (56). Whether telomeric location is directly associated also with singular contingency gene expression is unclear. Where the switch between different genes proceeds by promoter activation and deactivation, such as in *Plasmodium* or in trypanosome transcriptional switches, TPE has been invoked (25, 27, 28). However, early hopes that bloodstream VSG transcriptional switching was controlled by a TPE were not well supported by further experimental analysis (57), although a modified TPE was not ruled out. A degree of TPE of a reporter modified TPE was not ruled out. A degree of TPE of a reporter was more compelling because their promoters are only about 5 kb from the telomere tract and even be that evolution of the brief t-loop in trypanosomes has occurred as a means of divorcing telomere effects from the more exacting requirements of the VSG system.

**Acknowledgments**—We are grateful to Steve Jackson and Steve Bell (Wellcome/Cancer Research Campaign Institute, Cambridge) for discussions about Ku and in particular for opinions on the trypanosome sequences. We thank Piet Borst and Mike Cross (Netherlands Cancer Institute) for the gift of plasmids.

**REFERENCES**

1. Featherstone, C., and Jackson, S. P. (1999) *Mutat. Res.* 434, 3–15
2. Shore, D. (2001) *Curr. Opin. Genet. Dev.* 11, 189–198
3. Dubrana, K., Perroud, S., and Gasser, S. M. (2001) *Curr. Opin. Cell Biol.* 13, 281–289
4. Downs, J. A., and Jackson, S. P. (1999) *Mol. Cell. Biol.* 19, 6260–6268
5. Woodward, R. L., Lee, K. J., Huang, J., and Dynan, W. S. (2001) *J. Biol. Chem.* 276, 15423–15435
6. Aravind, L., and Koonin, E. V. (2001) *Genome Res.* 11, 1365–1374
7. Doherty, A. J., Jackson, S. P., and Weller, G. R. (2001) *FEBS Lett.* 500, 186–188
8. Milne, G. T., Jin, S. F., Shannon, K. B., and Weaver, D. T. (1996) *Mol. Cell. Biol.* 16, 4189–4196
9. Boulton, S. J., and Jackson, S. P. (1996) *EMBO J.* 15, 5093–5103
10. Porter, S. E., Greenwell, P. W., Ritchie, K. B., and Petes, T. D. (1996) *Nucleic Acids Res.* 24, 582–585
11. Manolis, K. G., Nimmo, E. R., Hartsuiker, E., Carr, A. M., Jeggo, P. A., and Allshire, R. C. (2001) *EMBO J.* 20, 210–221
12. Grandin, N., Damon, C., and Charbonneau, M. (2000) *Mol. Cell. Biol.* 20, 8397–8408
13. Petersen, S. E., Stellwagen, A. E., Diede, S. J., Singer, M. S., Haimberger, Z. W., Johnson, C. O., Tioneva, M., and Gutschovich, C. E. (2001) *Nat. Genet.* 27, 64–67
14. Ferreira, M. G., and Cooper, I. P. (2001) *Mol. Cell* 7, 55–63
15. Difilippantonio, M. J., Zhu, J., Chen, H. T., Medfe, E., Nussenzweig, M. C., Max, E. E., Ried, T., and Nussenzweig, A. (2000) *Nature* 404, 510–514
16. Baumann, P., and Cech, T. R. (2000) *Mol. Cell. Biol.* 11, 3265–3275
17. Gottschling, D. E., Aparicio, O. M., Billington, B. L., and Zakian, V. A. (1990) *Cell* 63, 751–762
18. Boulton, S. J., and Jackson, S. P. (1998) *EMBO J.* 17, 1819–1828
19. Baur, J. A., Zou, Y., Shay, J. W., and Wright, W. E. (2001) *Science* 292, 2075–2077

---

3. C. Conway, J. D. Barry, and R. McCulloch, unpublished results.
4. C. Conway, R. McCulloch, and J. D. Barry, in preparation.
Ku in Trypanosomes

21277

20. Vega-Palas, M. A., Venditti, S., and DiMauro, E. (1997) Nat. Genet. 15, 232–233

21. Moxon, E. R., Rainey, P. B., Nowak, M. A., and Lenski, R. E. (1994) Curr. Biol. 4, 24–33

22. Deitsch, K. W., Moxon, E. R., and Wellesms, T. E. (1997) Microbiol. Mol. Biol. Rev. 61, 281–284

23. Barry, J. D., and McCulloch, R. (2001) Adv. Parasitol. 49, 1–70

24. Rudenko, G., Cross, M., and Borst, P. (1998) Trends Microbiol. 6, 113–117

25. Scherf, A., Figueiredo, L. M., and Freitas-Junior, L. H. (2001) Curr. Opin. Microbiol. 4, 409–414

26. Stringer, J. R., and Keely, S. P. (2001) Infect. Immun. 69, 627–639

27. Horn, D., and Cross, G. A. M. (1995) Cell 83, 555–561

28. Rudenko, G., Blundell, P. A., Dirks-Mulder, A., Kieft, R., and Borst, P. (1995) Cell 83, 547–553

29. Barry, J. D., Graham, S. V., Fotheringham, M., Graham, V. S., Kobryn, K., and Wymer, B. (1998) Mol. Biochem. Parasitol. 91, 93–105

30. Cross, G. A. M. (1996) Bioessays 18, 283–289

31. Pays, E., Lips, S., Nolan, D., Vanhamme, L., and Perez-Morga, D. (2001) Mol. Biochem. Parasitol. 114, 1–16

32. Vanhamme, L., Pays, E., McCulloch, R., and Barry, J. D. (2001) Trends Parasitol. 17, 338–343

33. Robinson, N. P., Burman, N., Melville, S. E., and Barry, J. D. (1999) Mol. Cell. Biol. 19, 5839–5846

34. McCulloch, R., and Barry, J. D. (1999) Genes Dev. 13, 2875–2888

35. Donelson, J. E., Hill, K. L., and Elsayed, N. A. (1998) Mol. Biochem. Parasitol. 91, 51–66

36. Cross, G. A. M. (1975) Parasitology 71, 383–417

37. Hirumi, H., and Hirumi, K. (1989) J. Parasitol. 75, 985–989

38. Graham, S. V., Matthews, K. R., Shiels, P. G., and Barry, J. D. (1990) Parasitology 101, 361–367

39. Brun, R., and Schonenberger, M. (1979) Acta Trop. 36, 289–292

40. McCulloch, R., Rudenko, G., and Borst, P. (1997) Mol. Cell. Biol. 17, 833–843

41. Blundell, P. A., Rudenko, G., and Borst, P. (1996) Mol. Biochem. Parasitol. 76, 215–229

42. Gel, D., and Jackson, S. P. (1999) Nucleic Acids Res. 27, 3494–3502

43. Aravind, L., and Koonin, E. V. (2000) Trends Biochem. Sci. 25, 112–114

44. Walker, J. R., Corpina, R. A., and Goldberg, J. (2001) Nature 412, 607–614

45. Schwartz, J. (1989) Mutat. Res. 216, 111–118

46. Pays, E., Laurent, M., Delinte, K., Van Meirvenne, N., and Steiner, M. (1983) Nucleic Acids Res. 11, 8137–8147

47. Horn, D., Spence, C., and Ingram, A. K. (2000) EMBO J. 19, 2332–2339

48. Kass-Eisler, A., and Greider, C. W. (2000) Trends Biochem. Sci. 25, 200–206

49. Featherstone, C., and Jackson, S. P. (1999) Curr. Biol. 9, R759-R761

50. Lieber, M. R. (1999) Genes Cells 4, 77–85

51. Siede, W., Fried, A. A., Dianova, I., Eckardt-Schupp, F., and Friedberg, E. C. (1996) Genetics 142, 91–102

52. Mages, G. J., Feldmann, H. M., and Winnacker, E. L. (1996) J. Biol. Chem. 271, 7910–7915

53. Paques, F., and Haber, J. E. (1999) Microbiol. Mol. Biol. Rev. 63, 349

54. del Portillo, H. A., Fernandez-Becerra, C., Bowman, S., Oliver, K., Preuss, M., Sanchez, C. P., Schneider, N. K., Villalobos, J. M., Rajandream, M. A., Harris, D., da Silva, L. H. P., Barrell, B., and Lanzer, M. (2001) Nature 410, 839–842

55. Kitten, T., and Barbour, A. G. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6077–6081

56. Freitas-Junior, L. H., Bottius, E., Pirrit, L. A., Deitsch, K. W., Scheidig, C., Guinet, F., Nehrbass, U., Wellem, T. E., and Scherf, A. (2000) Nature 407, 1018–1022

57. Chaves, L., Rudenko, G., Dirks-Mulder, A., Cross, M., and Borst, P. (1999) EMBO J. 18, 4846–4855

58. Horn, D., and Cross, G. M. (1997) EMBO J. 16, 7422–7431

59. Griffith, J. D., Comeau, L., Rosenfield, S., Stanis, R. M., Bianchi, A., Moss, H., and de Lange, T. (1999) Cell 97, 503–514

60. Murti, K. G., and Prescott, D. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1436–1443

61. Munoz-Jordan, J. L., Cross, G. A. M., de Lange, T., and Griffith, J. D. (2001) EMBO J. 20, 579–588

Downloaded from http://www.bmc.org/ by guest on July 24, 2018
Ku Is Important for Telomere Maintenance, but Not for Differential Expression of Telomeric VSG Genes, in African Trypanosomes

Colin Conway, Richard McCulloch, Michael L. Ginger, Nicholas P. Robinson, Alison Browitt and J. David Barry

J. Biol. Chem. 2002, 277:21269-21277.
doi: 10.1074/jbc.M200550200 originally published online March 27, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M200550200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 61 references, 22 of which can be accessed free at http://www.jbc.org/content/277/24/21269.full.html#ref-list-1