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# Terminal region recognition factor 1, a DNA-binding protein recognizing the inverted terminal repeats of the pGKl linear DNA plasmids

**(gel retardation/protein-primed DNA replication/NFI/$\phi$29 p6 protein/pGKl killer plasmids)**

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ABSTRACT **The yeast linear DNA plasmids pGKl1 and pGKl2 contain inverted terminal repeats (ITRs) and terminal proteins covalently bound to the 5′ termini of each plasmid. The presence of these features suggests a protein-primed mechanism of DNA replication, similar to that exemplified by mammalian adenovirus and $\phi$29 phage of *Bacillus subtilis*. In this paper, we report the identification of an activity in cytoplasmic extracts of yeast harboring the pGKl plasmids that recognizes the termini of both pGKl1 and pGKl2. We call this activity TRF1, for terminal region recognition factor 1. Deletion analyses and DNase I protection experiments demonstrate that the activity recognizes base pairs 107–183 within the ITR of pGKl1, and base pairs 126–179 within the ITR of pGKl2. The presence of T-tracts within these two regions, but otherwise dissimilar nucleotide sequences, suggests that TRF1 recognizes a common structural feature within the ITRs of the two plasmids. TRF1 has been partially purified from yeast cytoplasmic extracts and Southwestern analysis indicates that the apparent molecular mass of the protein is 16 kDa. By expressing three open reading frames from pGKl2 in *Escherichia coli*, we found that open reading frame 10 (ORF10) of pGKl2 encodes TRF1. The sequence of the ORF10 gene product indicates that TRF1 is a highly basic protein of small molecular mass. Comparison of TRF1 with other DNA-binding proteins known to recognize the terminal regions of linear DNAs, such as NFI and NFIII involved in adenovirus DNA replication, and $\phi$29 p6, involved in $\phi$29 DNA replication, indicates that TRF1 has a different mode of binding.**

In recent years, a number of extrachromosomal linear DNA plasmids have been found in a variety of bacteria, fungi, and plants (1). The best characterized of these plasmids is the pGKl system, originally isolated in *Kluyveromyces lactis* as a pair of extrachromosomal DNA plasmids with a killer/immunity phenotype (2). Mutagenesis of a *K. lactis* strain containing the plasmids has resulted in either loss of both plasmids or loss of pGKl1 alone, suggesting that pGKl1 is dependent on pGKl2 for its maintenance (3). The smaller of these plasmids, pGKl1, is 8874 base pairs (bp) long (4, 5) with inverted terminal repeats (ITRs) of 202 bp (5). A 28-kDa protein is covalently bound to each 5′ terminus (6). pGKl1 contains an open reading frame (ORF) that encodes a putative DNA polymerase (7); genetic evidence suggests that this gene product is necessary for maintenance of the pGKl1 plasmid (8). The larger of the plasmids, pGKl2, is 13,457 bp long, has distinct ITRs of 184 bp (9), and covalently bound terminal proteins of 36 kDa (6). Like pGKl1, pGKl2 encodes a putative DNA polymerase (9). The presence of ITRs, terminal proteins, and plasmid-encoded DNA polymerases suggests a protein-primed mechanism of DNA replication (6, 10), by analogy with the $\phi$29 phage of *Bacillus subtilis* and mammalian adenoviruses.

The general model of protein-primed replication emerging from studies of adenovirus and $\phi$29 phage is that replication begins from either end of the linear genome, utilizing the viral-encoded DNA polymerase (11, 12). The polymerase catalyzes the addition of the first deoxynucleotide onto a terminal protein, this then serving as the primer for further elongation. In addition to the terminal proteins and plasmid-encoded polymerases, double-stranded DNA-binding proteins that recognize the terminal regions play important roles in the initiation of protein-primed DNA replication. In the case of adenovirus type 2, two host-encoded transcription factors, NFI (13–15) and NFIII (16), are required for efficient initiation of replication. Likewise, the p6 phage-encoded protein of $\phi$29 phage is required for efficient initiation of replication in this system (17). The presence of origin-binding proteins is widespread throughout other characterized replication systems as well. In *Escherichia coli*, a 52-kDa initiation factor, dnaA, binds at the origin and forms a nucleoprotein complex that permits melting of adjacent DNA, thus allowing entry of the replication complex (18). Initiation proteins of other systems, such as large tumor antigen in simian virus 40 (19) and O protein of $\lambda$ phage (20), play analogous roles by actively unwinding or destabilizing the adjacent duplex DNA.

In this paper, we report the identification of a DNA-binding protein, terminal region recognition factor 1 (TRF1), that specifically recognizes the termini of both pGKl plasmids. TRF1 is encoded by ORF10 of pGKl2, and the partially purified protein has an apparent molecular mass of 16 kDa. The small size and basic charge, as well as the DNA elements recognized by it, all suggest that TRF1 differs from NFI, NFIII, and p6 in its mode of interaction with DNA.

## METHODS

**Plasmids and Strains.** *Saccharomyces cerevisiae* strains AH22 [*MATa leu2-3,112, his4-519, can1*, (rho°), *cir*$^+$] and F102-2 [*MATa leu2-3,112, his4-519, can1*, (rho°), *cir*$^+$, pGKl1, pGKl2] were gifts from F. Hishinuma (Mitsubishi Kasei Institute of Life Sciences, Tokyo), as were plasmids pGKF107, pGKF106, pGKF201, and pGKF202 (5). pGKF107 and pGKF106 contain base pairs 1–4425 and 4426–8874 of pGKl1, respectively, cloned into the *E. coli–B. subtilis* shuttle vector pLS354. Plasmids pGKF201 and pGKF202 contain base pairs 1–5655 and 5656–13,457, respectively, of pGKl2 in the same pLS354 vector (5). The pKK480-3 *E. coli* expression vector contains the rat calmodulin gene under expression of the *trc* promoter (21) and was a gift of J. Brosius. Plasmid pBL107 was constructed by

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Abbreviations: TRF1, terminal region recognition factor 1; ITR, inverted terminal repeat; ORF, open reading frame.

cloning the 641-bp *Eco*RI/*Xba* I fragment from pGKF107 into pBluescript (Stratagene). Deletions of this plasmid were created according to the method of Henikoff (22).

**DNA-Binding Assays.** Gel retardation assays were performed essentially as described by Garner and Rezvin (23). Specifically, 1–50 fmol of $^{32}$P-labeled DNA probes (1000–5000 cpm) was incubated in the presence of 50 mM Tris·HCl, pH 7.4/5 mM $MgCl_2$/1 mM dithiothreitol/sonicated salmon sperm DNA (1 mg/ml), and various amounts of either yeast or *E. coli* cell extracts in a total vol of 25 μl for 20 min at 30°C. The reaction products were then resolved by agarose gel electrophoresis at 4°C and visualized by autoradiography. DNase I protection experiments were performed essentially as described by Galas and Schmitz (24).

**Purification and Characterization of TRF1.** Cytoplasmic extracts were prepared from stationary phase cultures by osmotic lysis of spheroplasts, after the method of Stark *et al.* (4). These extracts were then precipitated with 45–60% ammonium sulfate saturation. The precipitates were resuspended in buffer A, which contained 50 mM Tris·HCl (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 10% (vol/vol) glycerol, and were then separated by Sephacryl-300 column chromatography using buffer A. The TRF1 activity was pooled and separated by chromatography on native DNA-cellulose (Pharmacia) using a gradient of 50 mM to 1 M NaCl in buffer A. The active fractions were pooled and concentrated on an Amicon concentrator using a YM3 filter. Proteins were visualized by SDS/PAGE and Coomassie staining. Southwestern analysis was performed after the method of Miskimins *et al.* (25).

***E. coli* Expression of pGKl ORFs.** The calmodulin gene of pKK480-3 was excised by digestion of pKK480-3 with *Nco* I and *Xba* I and replaced with the coding sequences of pGKl2 ORF1, -5, or -10. These DNAs were amplified by PCR using primers that introduced an *Nco* I site at the first codon of each ORF and an *Xba* I site downstream of the termination codons. The resulting constructs, pKK2-ORF1, pKK2-ORF5, and pKK2-ORF10, were then used to transform *E. coli* cells. Expression of the gene products was induced with 0.15 mM isopropyl β-D-thiogalactoside and extracts were prepared as described by Marshall *et al.* (26).

## RESULTS

**Identification of a DNA-Binding Protein Specific for Terminal Sequences of the pGKl Plasmid DNAs.** To identify DNA-binding proteins that may be involved in the initiation of replication of the pGKl linear DNA plasmids, we used a gel retardation assay to look for factors that recognize the terminal sequences of the plasmids. Such a gel retardation was in fact observed when a DNA fragment containing 641 bp of the left terminus of pGKl1 was mixed with cytoplasmic extracts from F102-2 *S. cerevisiae* cells (Fig. 1, lane 3). A similar retardation was also observed with a probe containing the right terminus of pGKl1 (data not shown). In addition, retardation of a 246-bp fragment containing the left terminus of pGKl2 was observed in the presence of F102-2 extracts (lane 6), although the amount of the shifted fragment was less than that observed for a pGKl1 terminal fragment. These gel shifts were not seen when bovine serum albumin (lanes 1 and 4) or cytoplasmic extract from the parental AH22 strain (lanes 2 and 5) was used, suggesting that the DNA-binding activity is plasmid encoded or induced in the presence of the plasmids. All assays included 1 mg of sonicated salmon sperm DNA per ml, suggesting that the DNA-binding activity was specific for the terminal regions of the plasmids. In support of this conclusion, no slow-migrating species were observed when $^{32}$P-labeled internal fragments of pGKl1 (lanes 7 and 8) or pGKl2 (lanes 9 and 10) were used. In addition, neither poly(dI-dC) nor poly(dA-dT) was able to effectively compete for binding, whereas the binding to either radiolabeled terminal fragment was blocked by competition with unlabeled DNA containing either the pGKl1 left terminal fragment or the pGKl2 left terminal fragment (data not shown). These data suggested that the same activity recognized the terminal regions of both plasmids.

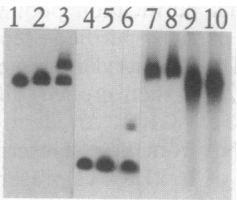


FIG. 1. Gel retardation assay with fragments from pGKl1 and pGKl2. $^{32}$P end-labeled fragments (2–5 fmol) were incubated at 30°C for 20 min with cytoplasmic extracts. The reaction mixtures were then electrophoresed on 1.8% agarose gels at 4°C, followed by autoradiography. The DNA fragments used were the 641-bp *Eco*RI/*Xba* I left terminal fragment from pGKF107 (lanes 1–3), the 246-bp *Eco*RI/*Cla* I left terminal fragment from pGKF201 (lanes 4–6), the 715-bp *Bam*HI/*Xba* I internal fragment from pGKF106 (lanes 7 and 8), and the 619-bp *Xba* I internal fragment from pGKF202 (lanes 9 and 10). The proteins used were 75 (lane 1) or 150 (lane 4) μg of bovine serum albumin, 75 (lane 2) or 150 (lanes 5, 7, and 9) μg of cytoplasmic extract from AH22, and 75 (lane 3) or 150 (lanes 6, 8, and 10) μg of cytoplasmic extract from F102-2.

**TRF1 Is a Protein with an Apparent Molecular Mass of 16 kDa.** TRF1 was partially purified from yeast extracts. Cytoplasmic extracts prepared from cultures of F102-2 by osmotic lysis of spheroplasts were fractionated by ammonium sulfate precipitation, where it was found that the TRF1 activity precipitated in the 45–60% saturation range. The ammonium sulfate precipitates were subsequently separated by gel filtration through Sephacryl-300. The majority of the activity eluted at a position where a marker protein of 12 kDa eluted (data not shown). This activity was pooled and further purified by chromatography on double-stranded DNA cellulose. TRF1 activity eluted between NaCl concentrations of 0.45 and 0.7 M.

SDS/PAGE and Coomassie staining revealed that the final preparation contained a major polypeptide with an apparent molecular mass of 16 kDa in addition to several minor larger molecular mass species (Fig. 2*A*). To determine which of the

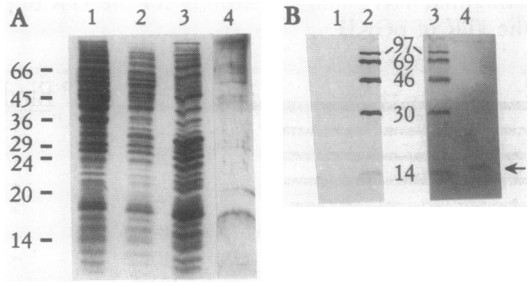


FIG. 2. Identification of TRF1 as a 16-kDa protein. (*A*) SDS/PAGE of TRF1 samples from various stages of purification. Samples were separated by SDS/PAGE and visualized by Coomassie staining. Lanes: 1, F102-2 cytoplasmic extract; 2, ammonium sulfate precipitate (45–60% saturation); 3, S-300 pool; 4, native DNA cellulose pool. Size markers (kDa) were purchased from Sigma (SDS-7). (*B*) Southwestern analysis. Concentrated preparations of TRF1 from native DNA cellulose were electrophoresed on SDS/12.5% polyacrylamide gels and transferred to nitrocellulose. Filters were then probed with a $^{32}$P-labeled 400-bp fragment ($10^5$ cpm/ml) containing the pGKl1 ITR (lanes 3 and 4) or a 173-bp fragment from pBluescript (lanes 1 and 2). Lanes: 1 and 4, ≈0.3 μg of TRF1 (DNA cellulose fraction); 2 and 3, $^{14}$C-methylated size markers (kDa) purchased from Amersham (CFA.626).

proteins in the final purification pool was responsible for TRF1 activity, the partially purified preparation was electrophoresed on an SDS/polyacrylamide gel, transferred to nitrocellulose paper, and then probed with $^{32}$P-labeled DNA containing the ITR of pGKl1. An identical blot was probed with $^{32}$P-labeled DNA from the pBluescript vector. As shown in Fig. 2*B*, a band of 16 kDa was present when the specific DNA probe was used but not when the nonspecific probe was used. UV-crosslinking of the DNA cellulose fraction to $^{32}$P-labeled DNA containing the ITR of pGKl1 also identified the 16-kDa protein as responsible for TRF1 activity (data not shown).

**TRF1 Recognizes a Region Within the ITRs of pGKl1 and pGKl2.** To identify more precisely the DNA sequence within the terminus of pGKl1 recognized by TRF1, 5′ and 3′ deletions were constructed within the pBL107 plasmid, which contained the leftmost 636 bp of pGKl1. The results are summarized in Fig. 3. Deletions from the 3′ end of pGKl1 containing at least the first 124 bp of the ITR were bound by TRF1, whereas fragments containing the first 107 bp or fewer did not. These results suggested that bp 108–124 within the ITR were important for TRF1 recognition. Deletions from the 5′ end of pGKl1 demonstrated that bp 116–636 could bind TRF1, whereas bp 171–636 could not, suggesting that the region between bp 116 and 170 contained an element(s) necessary for TRF1 recognition. Taken together, the deletion results indicate that TRF1 binds within the ITR of pGKl1, recognizing elements between bp 108 and 170.

DNase I protection analysis indicated that regions within the ITRs of both plasmids are recognized by TRF1. Fig. 4*A* illustrates that TRF1 protected a large region between approximately bp 107 and 183 on the top strand and bp 108 and 170 on the bottom strand of pGKl1. Full protection was seen at a protein concentration of 0.1 mg/ml, and no other regions of protection were seen even at a TRF1 concentration of 1 mg/ml. This result was supported by 1,10-phenanthroline copper footprinting, which showed protection in the region of bp 125–172 (data not shown). A similar region, with respect to distance from the terminus, was recognized in the ITR of pGKl2. Fig. 4*B* shows that with TRF1 concentrations of 1 mg/ml, protection from DNase I cleavage was observed in the region of bp 135–172 of the pGKl2 ITR on the top strand, and of bp 126–179 on the bottom strand. No clear protection was observed with a protein concentration of 0.1 mg/ml, suggesting that TRF1 had less affinity for the ITR of pGKl2 than the ITR of pGKl1.

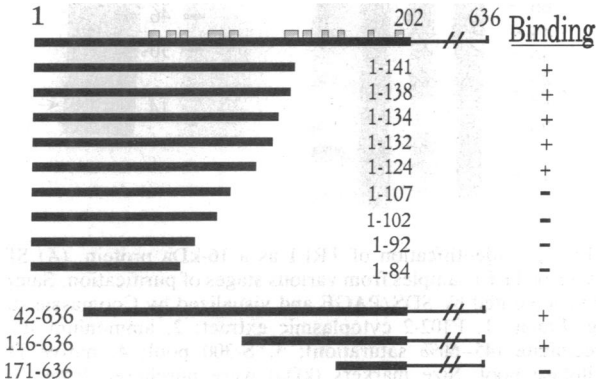


FIG. 3. Deletion analysis of TRF1 binding within the ITR of pGKl1. Deletions were constructed in the pBL107 plasmid. Lines represent pGKl1 sequences present in the plasmids, with the thick lines representing the ITR region. Hatched boxes indicate stretches of T residues longer than 3 nucleotides within the ITR. Binding was assayed by gel retardation using extracts from F102-2 cells. + indicates binding.

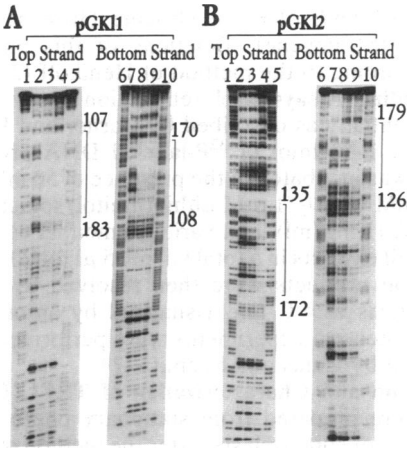


FIG. 4. DNase I protection of pGKl1 and pGKl2 ITRs. TRF1 binding reaction mixtures were incubated for 60 sec in the presence of DNase I (1 ng/ml). The products were then precipitated with ethanol, electrophoresed on denaturing polyacrylamide gels, and visualized by autoradiography. The TRF1 used was prepared by overexpression in *E. coli* with the pKK2-ORF10 construct. (*A*) DNase I protection of pGKl1 terminal fragment. (*B*) DNase I protection of pGKl2 terminal fragment. Lanes: 1, 5, 6, and 10, A+G chemical sequencing marker; 2 and 7, no TRF1; 3 and 8, TRF1 (0.1 mg/ml); 4 and 9, TRF1 (1.0 mg/ml).

**TRF1 Is Encoded by ORF10 of pGKl2.** The apparent size of TRF1 (16 kDa) as determined above (Fig. 2*B*) provided hints as to which ORF could encode it, assuming that TRF1 was plasmid encoded and not a plasmid induced host protein. Three of the small ORFs from pGKl2 that did not have any assigned function—ORF1, -5, and -10—were cloned by PCR into an *E. coli* expression vector, pKK480-3. *E. coli* transformants were induced with isopropyl β-D-thiogalactoside, and extracts were tested for binding activity by the gel retardation assay. As shown in Fig. 5*A*, extracts from *E. coli* expressing ORF10 gave the same gel shift of a probe containing the ITR of pGKl1 (lanes 12–14) as TRF1 purified from yeast (lane 2), whereas extracts from cells expressing no

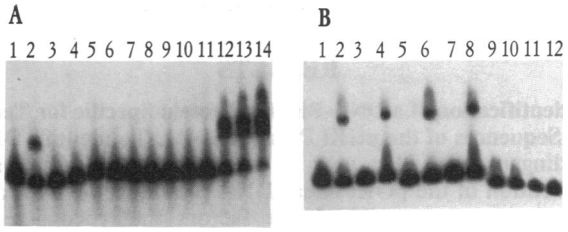


FIG. 5. Identification of ORF10 of pGKl2 as the gene encoding TRF1. (*A*) Expression of pGKl2 ORF10 in *E. coli* extracts gives specific retardation of a DNA fragment containing the pGKl1 ITR. Extracts from *E. coli* transformed with plasmids derived from the vector pKK480-3 expressing pGKl2 ORF1, -5, and -10 were prepared and tested for retardation of a $^{32}$P-labeled deletion fragment from pBL107, which contains bp 1–285 of the pGKl1 left terminus. The products were electrophoresed on a 1.5% agarose gel. Lanes: 1, no extract; 2, TRF1 from F102-2 (DNA cellulose fraction); 3–5, 1-, 2-, and 4-μl pKK480-3 extract; 6–8, 1-, 2-, and 4-μl pKK2-ORF1 extract; 9–11, 1-, 2-, and 4-μl pKK2-ORF5 extract; 12–14, 1-, 2-, and 4-μl pKK2-ORF10 extract. All extracts had similar protein concentrations of ≈7.5 mg/ml. (*B*) Gel retardation assay using pGKl1 terminal deletion fragments. $^{32}$P-labeled probes used were pGKl1 bp 1–138 (lanes 1–4), pGKl1 bp 1–124 (lanes 5–8), and pGKl1 bp 1–107 (lanes 9–12). Lanes: 1, 5, and 9, 146 μg of ammonium sulfate precipitate (45–60% saturation) from AH22 extracts; 2, 6, and 10, 146 μg of ammonium sulfate precipitate (45–60% saturation) from F102-2 extracts; 3, 7, and 11, 14-μg extract from *E. coli* transformed with pKK480-3; 4, 8, and 12, 14-μg extract from *E. coli* transformed with pKK2-ORF10. Electrophoresis was on a 2.0% agarose gel.

pGKl protein (lanes 3–5), ORF1 (lanes 6–8), or ORF5 (lanes 9–11) did not. At high TRF1 concentrations we have observed additional shifted bands in gel retardation studies as seen in lanes 12–14. These bands may be due to additional sites on the probe being occupied by TRF1 or higher-order protein–DNA complexes. To verify that the activity in the *E. coli* extracts expressing ORF10 was binding the same sequence as TRF1, we compared the *E. coli* extracts with F102-2 yeast extracts for binding to the deletions described above. It can be seen in Fig. 5*B* that the ORF10 gene product bound the same deletion constructs as the activity in F102-2 extracts. Specifically, the fragment containing bp 1–124 of pGKl1 was recognized by the TRF1 activity in yeast extracts (lane 6) and by the ORF10 gene product expressed in *E. coli* (lane 8). There was no DNA binding detected, however, when AH22 yeast extracts (lane 5) or extracts from *E. coli* carrying only the expression vector (lane 7) were used. The bp 1–107 fragment of pGKl1 did not show a gel mobility shift in the presence of extracts from ORF10-expressing *E. coli* (lane 12). Taken together, these results confirm that ORF10 encodes the TRF1 activity observed in yeast extracts.

## DISCUSSION

In this paper, we have reported the identification of a DNA-binding protein, TRF1, that recognizes the terminal sequences of pGKl1 and pGKl2 in the "killer" plasmid system in yeast. This protein is encoded by ORF10 of pGKl2 but recognizes the termini of both pGKl1 and pGKl2. The maintenance of pGKl1 is known to depend on the presence of pGKl2 (3) and, consequently, TRF1 may be one of the factors contributing to this dependency. We have also shown that ORF10 expressed in *E. coli* has TRF1 activity. This result, together with the Southwestern results, suggests that TRF1 alone is capable of binding to the DNA.

By deletion analysis of binding within the leftmost 636 bp of pGKl1, we found that TRF1 binds within the ITR. The regions between bp 107 and 183 of pGKl1, in particular, appear to be involved in TRF1 recognition, as demonstrated by deletion analysis as well as by DNase I protection. Fig. 4 shows that the same general region relative to the termini of each plasmid is protected from DNase I cleavage upon TRF1 binding. Comparison of the DNA sequences of these protected regions reveals that there is a prevalence of T stretches, although the actual DNA sequences are quite different (see Fig. 3). Some of these T stretches are repeated with a periodicity of ≈10 nucleotides, suggesting some bent structure (27), and it is tempting to speculate that TRF1 either binds DNA that is transiently bent, induces a particular bent DNA structure, or destabilizes the adjacent T-rich DNA to allow unwinding to take place. In fact, greatly exaggerated gel shifts were observed when the gel retardation assay was performed with polyacrylamide instead of agarose gel (data not shown), consistent with the observation that polyacrylamide is more sensitive than agarose gel to DNA structure (28). Attempts to demonstrate static bent structure within the ITRs, however, have so far been unsuccessful. The region between bp 60 and 82 contains three T stretches (see Fig. 3), and yet this element is not recognized in the deletions 1–107, 1–102, or 1–93. Similarly, poly(dA-dT) could not compete for binding, again suggesting that TRF1 is not merely recognizing dA-dT stretches. TRF1 presumably recognizes specific DNA sequence/structure characteristics that exist within the ITRs. Finer deletion and mutation analysis should reveal these sequence/structural requirements for binding.

The amino acid sequence of TRF1, deduced from the DNA sequence of pGKl2 ORF10, is shown in Fig. 6. The protein is highly polar, with a predominance of basic residues. The predicted molecular mass is 13.6 kDa, and the fact that it migrates slower on SDS/PAGE may be due to its overall basic charge. Tommasino (30) has recently reported that ORF10 of pGKl2 encodes a putative DNA-binding protein, based on its binding to DNA Sepharose. We report here that ORF10 is indeed a DNA-binding protein, but one that has specificity for the ITRs of pGKl1 and pGKl2. The small size and positive charge puts TRF1 in the larger classification of small, basic DNA-binding proteins of which histones and high mobility group proteins are members. However, we do not find in TRF1 the consensus sequences that have been identified for members of the high mobility group proteins (31) or other histone-like proteins from bacteria (32) or retroviruses (33). TRF1 may represent a separate family of small, basic DNA-binding proteins. The pSKl plasmid from *Saccharomyces kluyveri* has recently been sequenced, and the genome organization bears striking similarity to pGKl2 (29). The putative gene product of the ORF10 of pSKl is a small, basic protein of 105 amino acids. The regions of identity between TRF1 and the ORF10 gene product from pSKl are indicated by the boxed regions in Fig. 6 and suggest that these regions may contain common elements necessary for protein structure and/or DNA binding.

DNA-binding proteins that recognize the termini of linear DNAs have been found in the two well-characterized systems that utilize a protein-primed mechanism of replication, mammalian adenovirus and $\phi$29 phage of *B. subtilis*. In the adenovirus type 2 system, NFI binds a specific sequence within the ITR of the adenovirus genome (34) and stimulates replication initiation by protein–protein interaction with the adenovirus-encoded DNA polymerase (15) as well as by possible structural alteration of the origin DNA (35). NFIII, equivalent to the oct-1 transcription factor, also stimulates the initiation of adenovirus replication, again by possibly altering the structure at the origin (36). In the case of $\phi$29 phage, the p6 protein recognizes a region close to the termini of the linear $\phi$29 genome and binds cooperatively along the DNA, forming a nucleoprotein complex (17). Either the nucleoprotein structure or the DNA conformational change induced upon binding stimulates the initiation of DNA replication (37). TRF1 also binds near the termini of the pGKl plasmids, which contain covalently attached terminal proteins and ITRs, and consequently TRF1 may well play a role similar to NFI, NFIII, and p6 in the initiation of replication of the pGKl DNAs. The possibility that TRF1 acts in transcription is unlikely since each ORF has a putative promoter and is transcribed independently (4, 9), and the DNase I protection experiment in Fig. 4 did not detect any binding of TRF1 to the presumed promoter element. We have not, however, ruled out the possibility that TRF1 may be involved in the enhancement of transcription or in some other aspect of the biology of these plasmids.

Although NFI, NFIII, p6, and TRF1 are likely to play similar roles in DNA replication, the mode of their action may

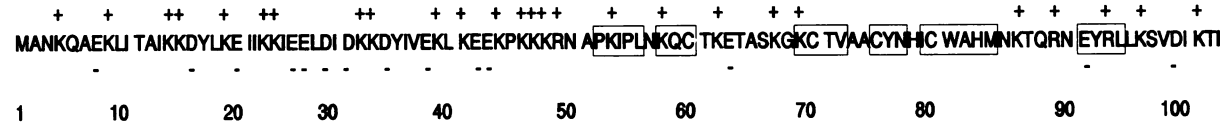


FIG. 6. Amino acid sequence of TRF1 deduced from ORF10 of pGKl2 (9). Charged residues are indicated + or −. Boxed regions indicate regions, >2 amino acids long, of identity between TRF1 and the ORF10 of the pSKl linear DNA plasmid of *S. kluyveri* (29).

be different. In contrast to NFI and NFIII, TRF1 is a small protein that does not exhibit DNA sequence-specific binding. In contrast to p6, TRF1 is a basic protein and does not form nucleoprotein complexes characterized by a repeated pattern of protection and hypersensitivity to DNase I cleavage (17). These comparisons suggest that at least three different types of initiation events might be utilized by protein-primed systems involving either site-specific DNA-binding proteins, nucleoprotein structures recognized by the replication machinery, or a DNA–protein complex exemplified by TRF1 binding. Further studies should reveal the function of TRF1 and the role of these DNA-binding proteins in the initiation of protein-primed DNA replication.

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