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Kinetoplast DNA minicircles of trypanosomatids encode for a protein product

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ABSTRACT

The major constituent of the trypanosomal kinetoplast DNA network are several thousand duplex DNA minicircles whose biological function is still unknown. The coding capacity and expression of these DNA minicricles, was studied in the trypanosomatid Crithidia fasciculata. Kinetoplast DNA minicircle fragments inserted into bacterial plasmid vectors were expressed in the bacterial cell. Sera elicited in rabbits, by immunization with the translational products of kinetoplast DNA minicircles in E. coli, reacted specifically with Crithidia fasciculata cellular antigens. It is inferred that kinetoplast DNA minicircles contain long open reading frames of nucleotides which are expressed in the trypanosomatid cell.

INTRODUCTION

Kinetoplast DNA (kDNA), the unique extrachromosomal DNA of trypanosomatids, consists in the species Crithidia fasciculata of about five thousand duplex DNA mincircles (2.5 kb each) and fifty DNA maxicircles (of about 37 kb) interlocked topologically to form a three-dimensional DNA network (reviewed in references 1-3). Although it has long been known to have an essential role in the life cycle of trypanosomatids and to be the cellular target for several trypanocidal drugs (4-6), the function of kDNA is still unknown. While it is widely agreed that maxicircles represent the mitochondrial DNA of this cell, the function of the major constituent of the network, the minicircle, is still puzzling. Based on their size, sequence heterogeneity, rapid evolution of their nucleotide sequences and their apparent lack of transcription (7-13), it has often been assumed that minicircles have no coding function. However, several observations suggested that further investigation of kDNA minicircle expression was merited. First, although minicircle transcripts have not been detected in L. tarentolae, C. luciliae, P. davidi and T. brucei (10-13), Fouts and Wolstenholme (14) have found an RNA transcript homologous to minicircles in C. acanthocephali. Although this RNA has not yet been characterized in detail, it appears to be of about 240

nucleotides in length, to anneal to the H-strand of the minicircle, but not to its L-strand, and to account for up to 1% of the total cellular RNA content. Second, direct analyses of nucleotide sequences of minicircles from three different trypanosomatids, T. brucei (15), T. equiperdum (16), and L. tarentolae (17,18) have revealed the presence of open reading frames of nucleotides in these DNA molecules. These sequences could potentially code for short polypeptide chains of about 20-70 amino acids in the different species. Translation initiation codons for these hypothetical translational products are located within a conserved sequence which is homologous in minicircles from two of the different trypanosomes species analyzed. Third, analyses of African trypanosomes defective in their kinetoplast DNA (reviewed in references 2,19,20) have suggested that their non viability in the insect vector (versus viability in the animal blood stream) is the result of deficient maxicircles and the loss of mitochondrial functions. However, since extensive genetic analyses of kDNA minicircle deficiencies have not yet been undertaken, the possible requirement for genetic information carried in kDNA minicircle sequences could not be excluded at this stage.

Here we have challenged the presumption that kDNA minicircles are not being transcribed and translated in the trypanosomatid cell, by assaying directly for open reading frames of nucleotides in the kinetoplast DNA minicircles and for the putative protein products encoded by such nucleotide sequences.

MATERIALS AND METHODS

Cell Growth:

E. coli strains MH1000 and TK1046, were a gift of Dr. G. Weinstock from the Genetic Engineering Laboratory NCI-Fredrick Cancer Research Facility, Fredrick, Maryland, and were grown as described by Weinstock et al. (21).

Crithidia fasciculata cell cultures were grown in Brain Heart Infusion (Difco) containing 20μg/ml Hemin (Sigma), 100 units/ml of penicillin and 100μg/ml streptomycin sulfate (Teva), at 28°C with shaking.

Nucleic Acids and Enzymes:

Kinetoplast DNA was prepared as described by Simpson and Simpson (22). kDNA minicircles were obtained by decatenation of purified kDNA networks using Crithidia type II topoisomerase (23) followed by preparative electrophoresis of the reaction products in 0.8% low melting agarose (Sigma) gels, and the isolation of the minicircles band from the gel by phenol extraction.

pORF1 and pORF2 were a gift of Dr. G. Weinstock and were prepared as described by Weinstock et al. (21). Restriction endonucleases, E. coli DNA

polymerase I and T4-DNA ligase were from New England Biolabs.

Preparation of Tribrid Fused Protein:

TK1046 cells containing pORF plasmids were grown at 28°C to OD_{600} = 0.2, and then at 38°C for 90 min. Cells were harvested by centrifugation and resuspended in solution containing SDS, glycerol, Dithiotreitol and bromophenol blue to final concentrations of 1%, 30%, 40mM, and 0.05%, respectively. Samples were loaded onto an SDS-polyacrylamide gradient (5-15%) gel and electrophoresed in 25mM Tris·Cl pH 8.4, 190mM glycin, and 0.1% SDS solution at 30V for 20 hrs at room temperature. The gel was stained using coomassie blue. Slices of gels containing the protein band were ground and used to immunize rabbits.

Antibody Preparations:

Rabbits were injected three times in two week intervals. 50ng protein samples were injected subcutanously at four different locations. Animals were bleeded before and after their immunization in order to obtain sera. These sera were adsorbed on extracts of bacterial cells producing the [ompF-lacZ]-fused protein product in order to adsorb antibodies directed against the ompF and lacZ protein components of the [ompF-kDNA-lacZ]-tribrid fusion. Each serum preparation was adsorbed 9 times on bacterial extracts polymerized as described by Avrameas and Ternynck (24).

Indirect Immunofluorescence Assays:

Cultures of Crithidia cells of 10^7 cells/ml were harvested and washed using phosphate buffered saline (PBS, 150mM NaCl and 10mM sodium phosphate pH 7.3). 10^7 cells were incubated at 40°C for 60 min with purified sera, then washed using PBS and incubated for 30 min at 4°C with fluorescent isothiocyanate conjugated (FITC)-goat anti rabbit immunoglobulin serum (Sigma). The cells were then washed three times with PBS, and immunofluorescence detected using fluorescent microscope or Fluorescence Activated Cell Sorter (FACS).

Immunoblotting Assays:

Immunoblotting assays were carried out as described by Burnette et al. (25). Extracts of bacterial or crithidial cells were electrophoresed in SDS-polyacrylamide gels. Protein bands were electroblotted onto nitrocellulose paper in solution containing 20mM Tris-base and 100mM glycine at 30V, at room temperature. Nitrocellulose papers were incubated in PBS containing 2% (w/v) bovine serum albumin for 1 hr and then washed twice in PBS containing 0.5% (w/v) bovine serum albumin, 0.05% (w/v) Tween 20 and 0.15% (w/v) Triton-X100. Incubations with immune or preimmune sera were

carried out for 90 min at room temperature. The papers were then washed, as above, and incubated for 60 min, at room temperature with ^{125}I-labelled S. aureus protein A (kindly provided by Dr. J. Hamburger, of this department). Immune complexes were detected by autoradiography.

Labelling of Crithidia Cells Using Radioactive Glucosamine:

20ml culture of Crithidia was grown to 4.3×10^7 cells/ml. The cells were washed and were further grown in the presence of 1 µci/ml ^{14}C-glucosamine (329.6 mci/mmol, Amersham) for 46 hrs. Cells were harvested, washed and lysed and the extracts were electrophoresed in polyacrylamide gels as described above. Gels were stained using coomassie blue, and prepared for fluorography, dried in vacuum, and autoradiographed as described by Laskey and Mills (29).

RESULTS

Open Reading Frames (ORF) Vectors Containing kDNA Minicircle Inserts.

Fragments of kDNA minicircles obtained by FnuDII and Alu I restriction endonuclease cleavage were inserted and ligated into the smaI site of the pORF expression vectors developed by Weinstock et al. (21) (Figure 1). The pORF vectors used (pORF-1 and pORF-2) contain the 5'-end of the E. coli ompF

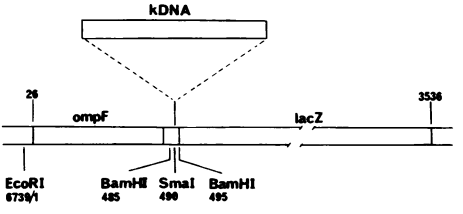

Figure 1

A schematic description of the molecular cloning of a kDNA minicircle fragment into a pORF expression vector. The pORF-1 and pORF-2 vectors described by Weistock et. al. (21) were used. The scheme describes the segment of the pORF-1 vector into which fragments of kDNA minicircles obtained by FnuDII and AluI restriction endonuclease cleavage were inserted and ligated at its smaI site. The realigning of ompF and lacZ genes in one reading frame resulting in the expression of β-galactosidase indicates that the kDNA fragment inserted is an open reading frame (see text for further details). Expression of the lacZ gene could also be induced by the excision of restriction fragment (BamHI to BamHI in pORF-1, or BamHI to BglII in pORF-2) (21), which also results in the realigning of ompF and lacZ in an open reading frame. These latter procedures were used for the construction of the bacterial clones used in control experiments and for the adsorption of antibodies directed against the ompF and lacZ encoded protein components of the ompF-kDNA-lacZ tribrid fusion. Site 3536 in the scheme is the end of lacZ gene.

gene, which provides a strong promoter, translation initiation site and a signal sequence for export from the cytoplasm. Coupled to it, but out of frame in respect to ompF is the E. coli lacZ gene that lacks its own 5'-end. kDNA derived fragments inserted between ompF and lacZ were found to support the realigning of ompF and lacZ genes in frame, which results in the expression of β-galactosidase activity in the cells. The lacZ phenotype thus

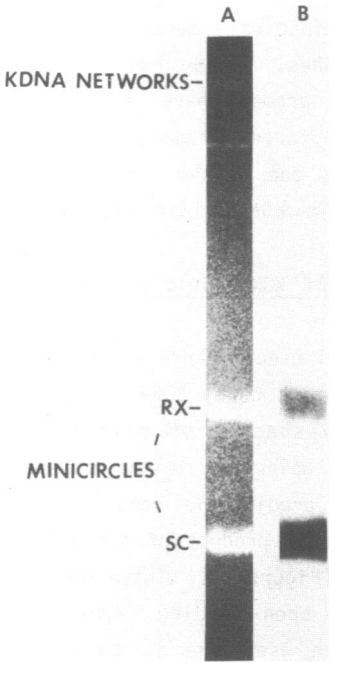

Figure 2

Molecular hybridization of decatenated kDNA networks with a kDNA insert rescued from the pORF2-2 vector. 1μg of kinetoplast DNA networks was decatenated using Crithidia fasciculata topoisomerase as described by Shlomai and Zadok (23). Reactions were stopped by the addition of SDS, glycerol, and bromophenol blue to final concentrations of 1%, 5% and 0.05% respectively. In A, reaction products were electrophoresed in 1% agarose gel containing 1μg/ml ethidium bromide (as described in Materials and Methods). DNA was denatured in the gel and transferred to nitrocellulose paper (26). IN B, a kDNA-fragment which was rescued from the pORF2-2 vector by BamHI and BglII endonuclease digestion (as described in the legend to Figure 1) followed by electrophoresis in 1% agarose gel and isolation form the gel (as described in the Materials and Methods), was labelled using (α-^{32}P)-ATP by nick translation (27). This ^{32}P-labelled DNA fragment was hybridized to the DNA products (photographed in A) bound to the nitrocellose paper as described by Wahl et al. (28). The paper was washed (28) and autoradiographed. RX and SC are the relaxed and the supercoiled forms of kDNA minicircles, respectively.

identifies clones containing an expressed open reading frame derived from the kDNA minicircle. Clones of bacteria that are lacZ and which were shown to contain kDNA minicircle sequences by colony hybridization to radioactive labelled kDNA minicircle probe were selected. Such kDNA inserts when rescued from the pORF vectors were found to hybridize specifically only with the minicircle component of decatenated kDNA networks (Figure 2). Restriction endonuclease analyses and molecular hybridization of the kDNA fragments inserted into the pORF vectors in the selected clones have confirmed their content of genuine kDNA minicircle sequences of 550 to 720 nucleotides in size in the different clones. These hybridization experiments, using the different kDNA inserts as probes, have revealed a close sequence homology between such inserts of different fragment sizes (not shown). The possibility that this homology is due to the molecular cloning of an open reading frame which is present in kDNA minicircles of heterogenous nucleotide sequences is investigated.

Long Open Reading Frames Of kDNA Minicircle Sequences Are Expressed In The Bacterial Cell.

SDS-polyacrylamide gel electrophoresis analyses of extracts of bacterial cells harboring plasmids which carry kDNA minicircle fragments have revealed the production of a new species of polypeptide chain in these cells (Figure 3). Its subunit molecular weight of about 135,000 daltons is in close agreement with the predictable mass of the tribrid protein fusion, expected as the translational product of the kDNA insert sandwiched between ompF and β-galactosidase (Figure 3). These observations indicate that kDNA minicircles contain long open reading frames that are expressed in the bacterial cell. We have used the E. coli TK1046 mutant to test for overproduction lethality as described by Weinstock et al. (21). Overproduction of [ompF-β-galactosidase]-fused proteins is lethal to the cell and this lethality is diagnostic for the initiation of translation within the ompF gene and its proceeding through the insert. Using this test, we have isolated cell clones in which the overproduction lethality indicates that the complete insert is being expressed as an open reading frame. In other clones isolated using this test, no such lethality was observed, indicating the presence of an ATG codon within the kDNA minicircle insert, which is in frame with lacZ. The precise location of the translation initiation site in the latter strains has yet to be determined. However, the size of the insert-translational product in these strains indicates that this initiation is close to the 5' terminus of the inserted fragment.

Antibodies Directed Against [ompF-kDNA-lacZ]-tribrid Fusion React With Crithidia Cell Antigens:

The new high molecular weight polypeptide, produced in significant amounts in the TK1046 cells, was extracted from polyacrylamide gels and injected into rabbits to raise antibodies against the fused tribrid protein product. Sera obtained were extensively adsorbed on polymerized extracts (24) of bacteria carrying pORF vectors that lack the kDNA insert, as described in the Materials and Methods. The bacteria used for this purpose were induced to experess β-galactosidase, by realigning the ompF and lacZ

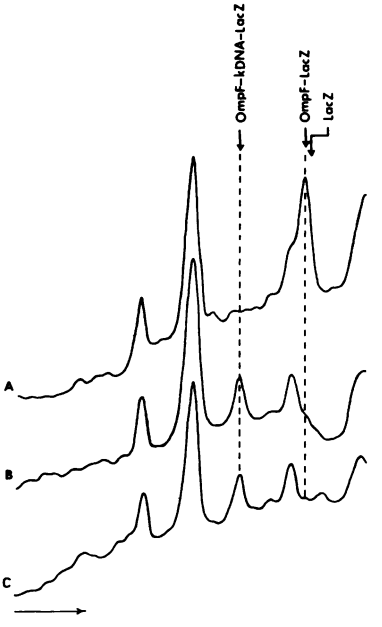

Figure 3

Microdensitometric presentation of an upper part of the SDS-polyacrylamide gel in which extracts obtained from three β-galactosidase producing strains of bacteria, were analyzed, as described in the Materials and Methods. Gel was stained using coomassie blue, dried in vacuum and scanned using a Quick Scan (Helana Laboratories) microdensitometer. In A, TK 1046 cells containing pORF-1 plasmid induced to produce β-galactosidase by the excision of a segment (as described in the legend to Figure 1) was used. In B and C TK1046 cells containing the pORF1-17 and pORF2-2 plasmids constructed by insertion of kDNA minicircle fragments into pORF-1 and pORF-2 (as described in Figure 1) were respectively used. LacZ indicates the migration in the gel of the E. coli β-galactosidase marker and ompF-lacZ and ompF-kDNA-lacZ that of the hybrid and tribrid fused protein products respectively. The arrow at the bottom indicates the direction of electrophoresis.

genes in frame (Figure 1), as described by Weinstock et al. (21). The treated rabbit sera were reacted with live crithidial cells in order to detect possible immune reactions between antibodies raised in the rabbit

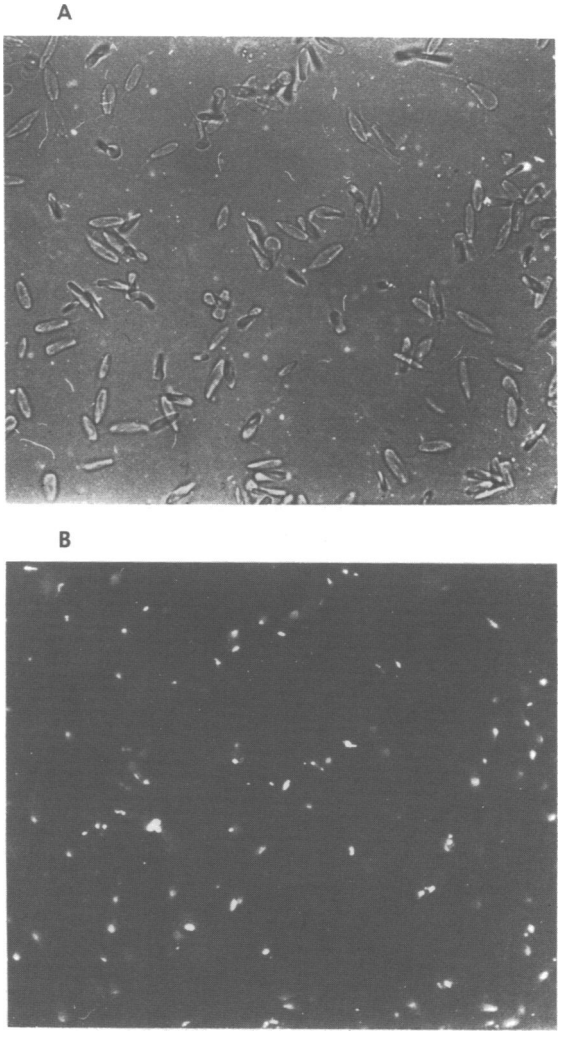

Figure 4
A culture of Crithidia fasciculata cells reacted with fluorescent antibodies specific for E. coli-kDNA tribrid fused protein. (A) A light microscopic view (x400); (b) A fluorescent view of the same field, showing the positively labelled cells (x400). The preparation of the (ompF-kDNA-lacZ)-fused protein product and sera, and the indirect immunofluorescence assay were as described in the Materials and Methods.

against the kDNA-E. coli fused protein and any crithidial cellular antigen. An indirect fluorescence assay, using goat antibodies directed against rabbit immunoglobulins, was used to detect possible immune complexes. Figure 4 shows that a live preparation of crithidial cells was found to react specifically with the rabbit immune sera, as indicated by their labelling with the FITC-goat anti-rabbit immunoglobulin serum. Essentially all the cells in the preparation displayed reactivity in the immunofluorescence assay and labelling was found to be associated mainly with the cellular site of the flagellum pocket. No such reactivity was observed in the immunofluorescence assay using sera obtained from these animals prior to their immunization. Thus, a specific immunofluorescence reaction was observed between antisera obtained from animals immunized with the (ompF-kDNA-lacZ)-encoded fused protein product of E. coli and Crithidia cells. Since antibodies directed against the E. coli, ompF and lacZ encoded protein components of the tribrid fusion were adsorbed out, these observations suggest a specific immune reaction between the antibodies directed against the kDNA-encoded translational product of E. coli and some crithidial cellular antigens. To further explore this possibility, we reacted the adsorbed immune sera with nitrocellulose paper onto which Crithidia extract proteins (or alternatively, those of E. coli cells which are either producers or non-producers of the tribrid protein) separated by SDS-polyacrylamide gel electrophoresis were blotted (Figure 5). Immune complexes formed were detected with ^{125}I-labelled S. aureus protein A, followed by autoradiography as described by Burnette et al. (25). No specific immune reaction could be detected when sera obtained from rabbits before immunization were reacted with extracts of bacterial cells producing the fused protein (not shown), or between immune sera and extracts obtained from non-producers (Figure 5). However, a specific immune complex is formed when immune sera are reacted with extracts of bacterial cells producing the tribrid fused protein. When a similar protocol was applied to Crithidia cell extracts, it was found that sera obtained from immunized rabbits reacted specifically with three polypeptide chains present in these extracts (Figure 5). No such reaction could be detected using sera from animals before immunization. Considering the size of the intact kDNA minicircle, the observed electrophoretic mobilities of these proteins in polyacrylamide gel was lower than anticipated. This could be attributed to their content of sugar residues, as two of these polypeptide chains were efficiently labelled with radioactive glucosamine (not shown). A detailed characterization of these polypeptide chains as glycoproteins is now in progress.

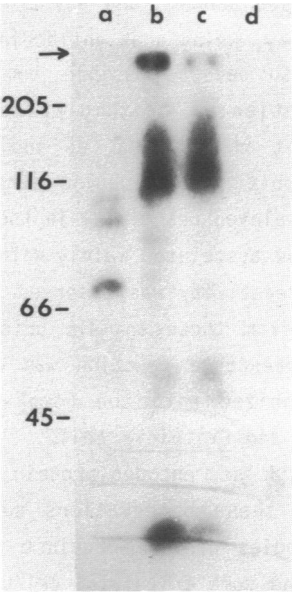

Figure 5
Immune reactions of antibodies directed against the [ompF-kDNA-lacZ]-fused protein with cell extracts proteins. Immunoblotting assays were carried out as described in the Materials and Methods. Antiserum directed against pORF1-17 derived tribrid fusion (adsorbed as described in the Materials and Methods), was reacted with extracts of the E. coli tribrid protein producers: TK1046 carrying pORF1-17 (b) and pORF2-2 (c) and a non producer (TK1046 carrying the pORF-1 which produces the hybrid [ompF-lacZ]-fused protein as described in the legend to Figure 1) (d). In (a) the adsorbed serum is reacted with Crithidia fasciculata extract proteins. The numbers represent the molecular weights of protein markers ($x10^{-3}$): myosin (205,000), β-galactosidase (116,000), bovine serum albumin (66,000) and ovealbumin (45,000). The top of the gel is marked by the arrow.

DISCUSSION

The presumption that kDNA minicircles are not transcribed in the trypanosomatid cell was based upon previous observations reported by several laboratories of the lack of detectable RNA transcripts homologous to these DNA molecules (10-13). However, if transcription of kDNA minicircles is limited to a specific short period during the hemoflagellate life cycle, or if the mRNA transcripts are not abundant or extremely unstable, they might not have been detected in these studies. For these reasons, this research was designed to detect open reading frames in the kDNA minicircle and putative protein products encoded by such nucleotide sequences, circumventing the need for detecting RNA transcripts.

The results reproted here indicate the presence of open reading frames of nucleotides in the major constituent of the kinetoplast DNA, the minicircle, and suggest the possibility of their expression in the Crithidia cell. This conclusion is based upon the expression of kDNA minicircles sequences in the bacterial cell (Figure 3), and the specific immune reaction observed between antibodies raised against the translational products of such sequences in E. coli and Crithidia cellular antigens (Figures 4,5).

The observation reported here that Crithidia fasciculata kDNA minicircle contains long open reading frames of nucleotides is in agreement with the sequencing data reported on T. brucei (15), T. equiperdum (16) and L. tarentolae (17,18), which indicated the presence of an open reading frame common to minicircles of a general heterogenous nucleotide sequences. These sequences were also found to contain a potential site for initiation of translation. In this respect, the pORF vectors described here in which overproduction-lethality (21) suggests translation initiation from a site within the minicircle insert, are especially interesting. Whether the crithidial translation system actually uses these signals, which are recognized in the bacterial cell, has yet to be determined. However, the observations reported here provide the first experimental data indicating the existence of kDNA minicircle encoded protein products in trypanosomatid cells. The nature of these products and their role in the life cycle of the cell is yet unknown. Labelling experiments using radioactive glucosamine suggest that these products might be glycoproteins. Content of sugar moieties might account for their apparent migration as high molecular weight polypeptide chains in the SDS-polyacrylamide gel (Figure 5). We believe that further characterization of these translational products as well as further investigation into the potential coding capacity and the expression of the kDNA minicircle will shed some light on the biological function of this bizarre DNA entity of the kinetoplast DNA in the trypanosomatid cell.

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