
A new type of plasmid from a wild isolate of *Dictyostelium* species: the existence of closely situated long inverted repeats

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ABSTRACT

A circular plasmid having high copy number was found in a wild isolate of *Dictyostelium* species. Gel electrophoresis, electron microscopy and Southern blot hybridization revealed that the plasmid, named pDG1, is 4.5Kb(1.5 μ m) in size with closely situated long inverted repeats. The plasmid seems to be located in the nuclei. It was not a derivative of ribosomal DNA. The possible correlation of the plasmid with the putative intermediate DNA of retrotransposon DIRS-1 found in *Dictyostelium discoideum* is discussed.

INTRODUCTION

The cellular slime mold, *Dictyostelium discoideum* is an ideal material for use in studies on fundamental problems such as development and cell motility(for review, 1). Recently, the structure and developmental expression of the several *Dictyostelium* genes have been examined using recombinant DNA techniques and results have indicated several interesting features of the genomic organization and development of *Dictyostelium*(2-6).

In *D. discoideum*, several plasmids have been isolated and the molecular characterization have been reported about one of the plasmids, Ddp1(7-9). Furthermore, Ddp1 was used successfully for the construction of the shuttle vector pDdp1-20 in *D. discoideum* with the combination of an integration vector B10S(10). The B10S contains the bacterial Tn5 neomycin resistance gene flanked by *D. discoideum* actin 6 promoter region and actin 8 poly A signal (11).

We also attempted to find plasmids in wild isolates of the cellular slime molds to construct a vector in these organisms. In this paper, we report the finding and some characteristics of a new closed circular DNA, named pDG1, having long inverted repeats which are closely situated, in a *Dictyostelium* species isolated from soil. Recently, Cappello *et al.*(4) reported the complete sequence of an apparent retrotransposon DIRS-1 with long inverted terminal repeats in *D. discoideum* and proposed the biosynthetic

route of a putative circular, transposon intermediate DNA. We discuss our results in terms of the possible correlation between the plasmid pDG1 and the putative transposon intermediate DNA.

MATERIALS AND METHODS

Organisms and growth of amoebae. The laboratory strains used were Dictyostelium discoideum NC4(mat A1, from Dr.M.Sussman) and V12(mat a2, from Dr.J.T.Bonner) and Dictyostelium mucoroides(Dm7, from Dr.Y.Maeda). The wild isolates(65 isolates) of Dictyostelium species were obtained from soil from several parts of Japan. All strains were grown at $21\pm 1^{\circ}\text{C}$ in association with Klebsiella aerogenes on SM agar(12).

Isolation of plasmid DNA. Plasmid DNA was isolated from the cellular slime molds by the alkali-SDS method(13). For removal of bacteria, cells in the growing phase collected from 10 petri dishes(2×10^9 cells) were washed 7 times with cold Bonner's salt solution(NaCl, 0.6g; KCl, 0.75g; CaCl_2 , 0.3g per liter) by centrifugation at 2000rpm for 2min. The cell pellet was suspended in 30ml of cold plasmid extraction buffer[PE buffer; 25mM Tris-HCl (pH 8.0), 10mM EDTA, 50mM glucose]. Then 60ml of alkali-SDS solution(0.1N NaOH, 1%SDS) was added and the solution was allowed to settle at room temperature for 30min. Then 45ml of 3M sodium acetate(pH 6.0) was added with gentle mixing and the mixture was kept on ice for one hour. It was then centrifuged at 10,000rpm for 30min. The precipitate was dried and dissolved in 5ml of TE buffer[10mM Tris-HCl(pH 7.6), 0.1mM EDTA]. For screening, the DNA was purified by extraction with TE buffer saturated phenol three times and precipitation with ethanol, and dissolved in 0.1ml of TE buffer. Then, the DNA was analysed by 0.8% agarose gel electrophoresis. For molecular characterization of the plasmid, crude plasmid DNA from 100 petri dishes was further purified using banding method in CsCl density gradients with ethidium bromide(EtBr)(14). In the case of K. aerogenes, the procedures were exactly as described by Maniatis et al.(15).

Preparation of nuclear DNA. Nuclei were prepared as described by Firtel and Lodish(17). The DNA was isolated from the nuclei(14). Total DNA of K. aerogenes was prepared as described by Davis et al.(18).

Electron microscopy of plasmid. The purified, closed circular DNA obtained from CsCl banding were freeze-thawed several times to introduce a nick in the molecules. The DNA was then spreaded, and shadowed as described by Kleinshmidt et al.(19) and was observed under a JOEL 100C electronmicroscope at an accelerating voltage of 80 kilo electron volts. For visualiza-

tion of repeated sequences, electronmicroscopical homoduplex experiments were done exactly as described(20).

Restriction enzyme digests and gel electrophoresis. Various restriction endonucleases were used under the conditions recommended by the suppliers. The digests were fractionated on 0.8% or 1.0% agarose gel in Tris-aetate-EDTA buffer(pH 8.0) and on 6% or 8% polyacrylamide gel in Tris-borate-EDTA buffer(pH8.0)(15). DNA was stained with EtBr. For the elucidation of the existence of inverted repeats, the pDG1 DNA was digested with XmaIII and was labelled with [α -³²P] dGTP by fill-in reaction using Klenow fragments(15). The products were fractionated on a 4.5% polyacrylamide gel and the gel was autoradiographed. Densitometry was performed using a Beckman DU-8B spectrophotometer equipped with slab gel scanning system.

Southern blot hybridization Plasmid DNA as a probe was labelled in vitro with [α -³²P] dATP by nick translation(21). The specific activity of the DNA was 1-5 x 10⁸ cpm/ μ g. DNA was size fractionated on gels and blotted on the GeneScreen PlusTM filter as described by the supplier. The hybridization procedures by Maniatis et al.(15) were followed with modifications. The filters were prehybridized at 42°C for 6h with a solution containing 50% formamide, 10% dextran sulfate, 5 x Denhardt's solution, 6 x SSC(1 x SSC; 0.15M NaCl, 0.015M sodium citrate), 0.5% SDS and 0.5mg/ml of denatured salmon sperm DNA. The hybridization solution was the same, except for the 0.1mg/ml of denatured salmon sperm DNA and for the inclusion of 100ng of labelled DNA. Hybridization was at 42°C for 20h. The filters were then rinsed at 22°C in 2 x SSC twice and washed at 65°C in 2 x SSC/ 0.1% SDS for several hours twice. After air drying the filter were exposed at -80°C with intensifying screens overnight.

Enzymes and chemicals. Restriction enzymes were purchased from Takara Shuzo Co., Toyobo Co., Boehringer Mannheim and Bethesda Research Labs. E.coli DNA polymerase I and DNase I were from Takara Shuzo Co.. GeneScreen PlusTM was from NEN Research Products. DEAE-Sephacel was from Pharmacia. [α -³²P] dATP(3000 Ci/mmole) and [α -³²P] dGTP(3000 Ci/mmole) were obtained from Amersham. All other chemicals were special grade.

RESULTS

Plasmid screening

As shown in Fig.1a (lane 4), a discrete band was discovered in the electrophoretogram of one strain, named GA11, out of 65 of the wild isolates, but no band was found in the laboratory strains, NC4 and V12(lanes 2 and 3).

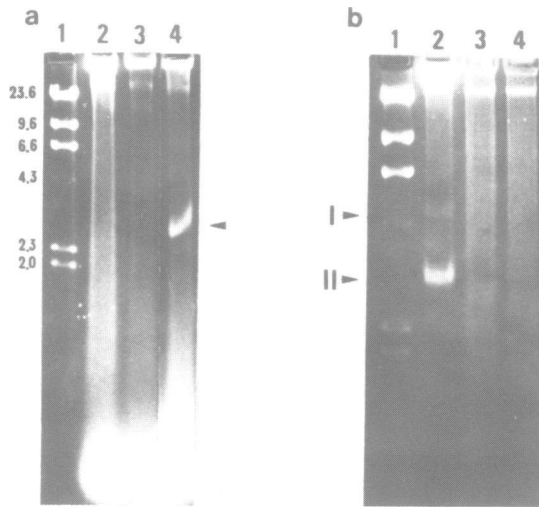


Fig.1.a. Agarose gel electrophoresis of DNA extracted from *Dictyostelium* sp. DNA extracted from *D. discoideum* strain NC4(lane 2), strain V12(lane 3) and the wild isolate, strain GA11(lane 4). The samples were not treated with RNase A. Lane 1 is phage λ DNA digested with HindIII. One lane corresponds to 5×10^8 cells and the arrow indicates the plasmid band. **b.** Agarose gel electrophoresis of nuclear DNA from *Dictyostelium* strain GA11(lane 2) and *K. aerogenes*(lanes 3 and 4). DNA was extracted with(lane 4) or without(lanes 2 and 3) lysozyme treatment. Lane 1 is phage λ DNA digested with HindIII. For explanation of bands I and II, see text.

The wild strain GA11 has unbranched stalk (Fig.2) and produces non-migrating pseudoplasmodia. The fruiting bodies are white and do not have disc. The spores are ellipsoidal and have no refractile body. Amoebae are chemotactic to cAMP. According to Raper's system of classification(16), this GA11 strain failed to fit into any key for *Dictyostelium* at the species level. For the precise classification of the strain, further investigations would be necessary, especially on molecular level.

Plasmid is not of bacterial origin.

Since the *Dictyostelium* species was grown on a lawn of *K. aerogenes* as food, the plasmid might have been derived from the bacteria. To examine this possibility, we attempted to extract the plasmid from the bacteria by two different methods; that is, the same procedure as for the plasmid preparation from *Dictyostelium* strain GA11(Fig.1b lane 3) and a similar method but with additional treatment of the bacteria with lysozyme(1mg/ml) in PE buffer(Fig.1b lane 4). *K. aerogenes* cells(total 10^{11} cells per lane) gave no detectable band.

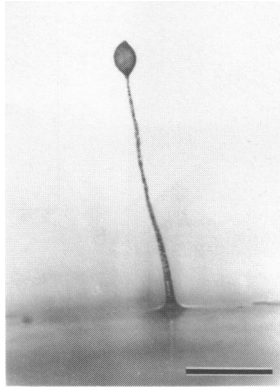


Fig.2. The final structure of fruiting body of the wild isolate, Dictyostelium species strain GA11. Bar=0.5 mm.

Plasmid is closed circular, double stranded DNA and 4.5Kb long.

The plasmid preparation of strain GA11 gave two bands(bands I and II) of different band intensities on agarose gel stained with EtBr(Fig.1b lane 2). When band II was treated with restriction enzymes EcoRI or PstI, only band I appeared(data not shown). Fractions in the denser region on CsCl-EtBr equilibrium density gradient centrifugation were found at band II on electrophoresis(data not shown). Band I was found to be about 4.5Kb long in the electrophoretogram. Fig.3a shows closed and open circular forms of DNA in the plasmid preparation. The size frequency distribution is presented in Fig.3b and the plasmid is measured as 4.5Kb long. This value is the same as that obtained by agarose gel electrophoresis. We named the plasmid pDG1.

Restriction enzyme mapping and homoduplex analysis.

For constructing the physical map of pDG1, the DNA was digested with various restriction enzymes. The relative positions of restriction enzymes sites were determined by agarose and polyacrylamide gel electrophoresis of double digests(Fig.4a). AatI, AatII, BamHI, PvuII, SalI and XbaI did not have cutting site. Restriction sites of plasmid pDG1 differed from that of the plasmid Ddp1(9). In addition, ClaI and XmaIII cutting sites show the possible existence of inverted repeats in pDG1. Furthermore, when the plasmid pDG1 was digested with XmaIII, filled in with [α -³²P] dGTP and electrophoresed on a 4.5% polyacrylamide gel, the XmaIII-XmaIII fragments showed twice the intensity of band(band B) compared to XmaIII-ClaI-ClaI-XmaIII fragments(band A)(Fig 4b). To examine this possibility more precisely,

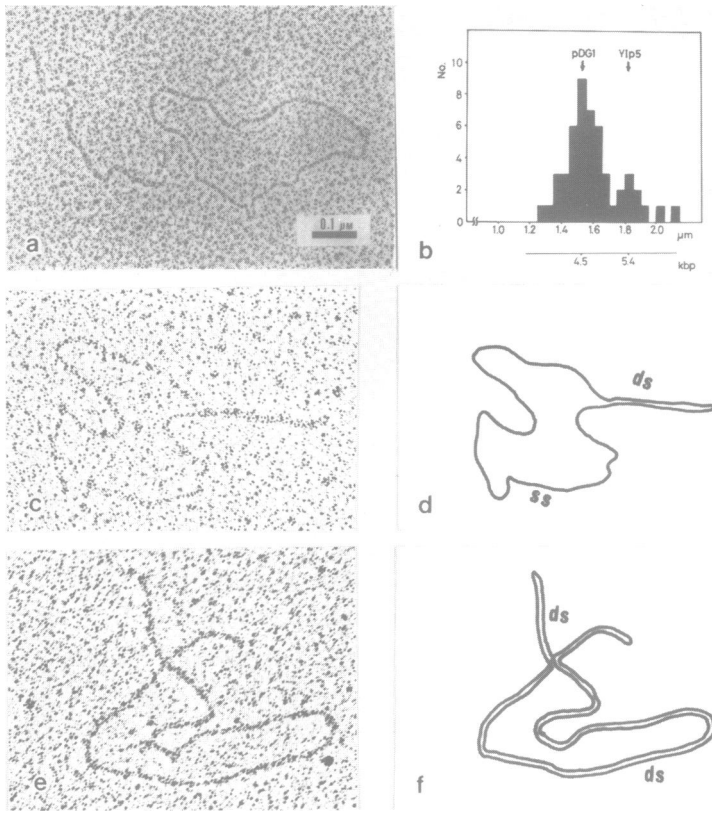


Fig.3. a. Electron micrograph of plasmid pDG1. **b.** Size distribution of plasmid pDG1. Fifty samples were examined. Yeast plasmid YIp5(1.8μm, 5.4Kb) was used as an internal standard. **c** and **e.** Homoduplexes of pDG1. **d** and **f.** The tracing of the homoduplexes. ss; single strand, ds; double strand.

homoduplex analysis was done. Fig.3(c and d) shows "round fan" like structure of pDG1 after intrastrand annealing. The length of the inverted repeats was calculated as 560 ± 50 bases (N=16) using double stranded open circular DNA in the same specimen as a standard. When two complementary strands were annealed, two looped-out rods were present (Fig.3 e and f). In this case, the length was 550 ± 30 bases (N=16). The frequency with "round fan" like structure was 30 % and in the case of two looped-out rod structure, it was also 30 %.

Southern blot hybridization experiments with pDG1.

BglII digest of pDG1 of strain GA11 gave only one band at around 4.5Kb region with the labelled pDG1 (Fig.5a. lane 2). Moreover, the radioactive band did not coincide with any restriction fragments derived from ribosomal

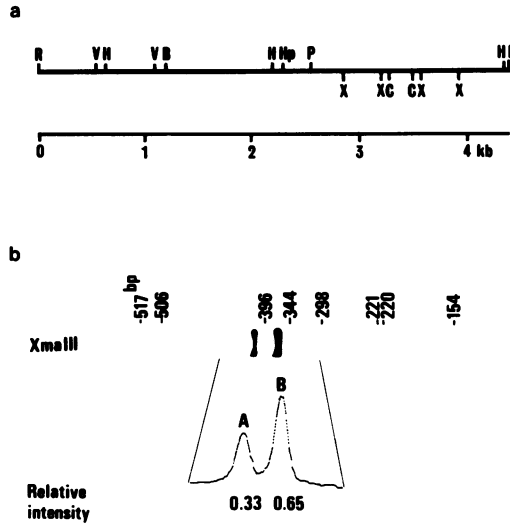


Fig.4. a. Restriction map of plasmid pDG1. B; BglIII, C; ClaI, H; HindIII, Hp; HpaII, P; PstI, R; EcoRI, V; EcoRV, X; XmaIII. Note that BglIII, EcoRI, HpaI and PstI have only one cutting site. **b.** Evidence for inverted repeats in pDG1. The XmaIII-XmaIII fragments of the plasmid pDG1 repeats exist twice as much as other ragment. upper: Autoradiogram. lower: The autoradiogram was scanned using a Beckman DU-8B slab gel scanner.

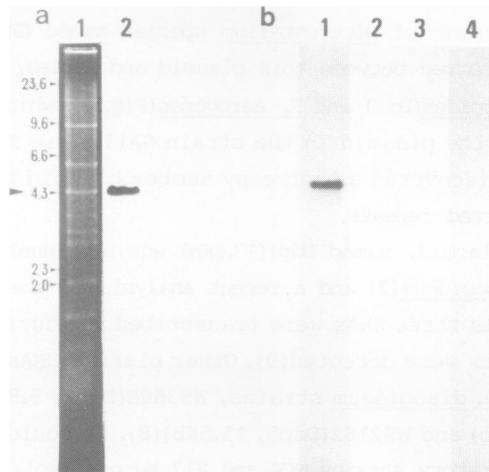


Fig.5. a. Southern blot analysis of BglIII digested nuclear DNA (*Dictyostelium* strain GA11) with labelled pDG1 DNA. Lane 1 was stained with EtBr. The arrow indicates the linear plasmid band(4.5Kb). The same gel was subjected to blotting and autoradiography as mentioned in text. **b.** Autoradiogram of Southern blot analysis. BglIII digested nuclear DNAs(5µg) from different organisms were used. *Dictyostelium* species strain GA11(lane 1); *D. discoideum* NC4(lane 2); *D. mucoroides*Dm7(lane 3); *Klebsiella aerogenes*(lane 4).

DNA of GA11 strain which were seen as discrete bands on the EtBr stained gel. That means pDG1 has no sequences which were homologous to genomic DNA of GA11 strain. However, it is present in at least the nuclei. The probe did not hybridize to nuclear DNA from D. discoideum(NC4), D. mucoroides(Dm7) and K. aerogenes(Fig.5b lanes 2-4). Southern hybridization mentioned above was also done with EcoRI digests of pDG1 and nuclear DNA of these organisms and similar results were obtained(data not shown).

Copy number of pDG1.

Noegel et al.(8) estimated the copy number of their plasmids from the comparison with the band intensity of the extrachromosomal ribosomal DNAs which exist at 180 copies per cell in the strain NC4. Although we have not yet estimated the copy number of the ribosomal DNA of GA11 strain, the comparison of the band intensities on the gel(Fig.5a lane 1) would allow us to say that pDG1 exists in roughly the same number as that of the ribosomal DNA, that is, as high copy number.

DISCUSSION

For construction of vectors in the cellular slime molds, we searched for plasmids in about sixty wild isolates. As shown in this report, a novel circular plasmid DNA with 0.5-0.6Kb long inverted repeats, pDG1(4.5Kb), was found in one wild isolate of Dictyostelium species named GA11. No Southern hybridization was observed between this plasmid and nuclear DNA of D. discoideum(NC4), D. mucoroides(Dm7) and K. aerogenes(Fig.5), which indicates the unique existence of the plasmid in the strain GA11. This is for the first time that has been discovered a high copy number plasmid bearing closely situated "long" inverted repeats.

An endogenous plasmid, named Ddp1(13.8Kb) was previously found in several strains of D. discoideum(7) and a recent analysis of the plasmid showed that in growing cells three RNAs were transcribed and during development five more transcripts were detected(9). Other plasmid DNAs have been also found in wild type D. discoideum strains, WS380B(Ddp2, 5.8Kb), OHIO(Ddp3, 27Kb), AC4(Ddp4, 22Kb) and WS2162(Ddp5, 15.5Kb)(8). We could not detect any plasmids in the laboratory strains NC4 and V12 by our isolation procedures. This is in contrast to the results of Metz et al.(7), who found the plasmid Ddp1 in the NC4 and V12 strains. Plasmid Ddp1 may have been cured in our laboratory stocks since the strains harboring Ddp1 were both early stocks lyophilized in 1954 in K.B.Raper's laboratory(in ref. 7).

Metz et al.(7) suggested that Ddp1 may be correlated with cobalt

resistance. At present, we do not have any information about the characteristics, such as drug resistance, of pDG1.

As the method for preparation of the pDG1 DNA from strain GA11 was laborious and the yield was low, we attempted to clone the DNA into pBR322 or YIp5 using the EcoRI or PstI site. The recombinant DNAs transformed in E. coli HB101 or C600, and isolated from the transformants had a deletion of about 1Kb or less(data not shown). The unique structure of pDG1 may be responsible for the inability of the fused DNA to persist in E. coli strains HB101 or C600. The instability of inverted repeats in E. coli was already reported(22, 23).

Recently, Cappello et al.(4) reported the total sequence of DIRS-1, an apparent transposon in D. discoideum. DIRS-1 consists of 4.1Kb of internal sequence flanked by long inverted terminal repeats of unequal lengths(24). The DNA sequence analysis had revealed that it has three open reading frames(ORFs) in the 4.5Kb RNA transcript and a 200 amino acid region of ORF3(2007 bp) has significant homology to retrovirus reverse transcriptase(4). They proposed the synthetic route of a putative transposon intermediate DNA(4). The molecular features of this intermediate DNA are very similar to that of pDG1. Although the two strains should be different species and the two circular DNAs have different restriction maps, it is interesting to suppose that pDG1 is a transposon intermediate DNA or its relative in the GA11 strain. Some questions arise here. First, the copy number of pDG1 is very high compared to other circular transposable elements(25-27). Second, even under careful experiments, Southern blot analysis did not show any hybridizable bands derived from genomic DNA. These results are unfavorable to the assumption. Even if, the pDG1 is not an intermediate DNA, it is conceivable that the plasmid could have been an intermediate bearing a replication origin, which lost the integration activity into genomic DNA in the past, and then might have existed as an autonomously replicating cellular element up to the present. In this context, it is interesting to note that in a mitochondrial plasmid of the Mauriceville-Ic strain of Neurospora crassa, the plasmid-encoded protein contains related sequences to several reverse transcriptases of retroviruses(28) but lacks corresponding sequences of DNA endonucleases(29). To get further information concerning this assumption, DNA sequencing and analysis of RNA transcripts of pDG1 are essential.

Our initial purpose is to construct vectors of D. discoideum. As the strain GA11 is not D. discoideum, there may be a phylogenetic species barrier to the construction. Even if pDG1 does not act as a vector in D. discoi-

deum, it may be a good DNA model for use in studies on the characteristics of eukaryotic extrachromosomal DNAs.

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