Insertion of transformation vector DNA into different chromosomal sites of *Dictyostelium discoideum* as determined by pulse field electrophoresis

Robert A. Cole and Keith L. Williams

School of Biological Sciences, Macquarie University, Sydney 2109, NSW, Australia

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ABSTRACT

Chromosomes of the cellular slime mold *Dictyostelium discoideum* were fractionated on three pulse field gel electrophoresis systems (pulse field, orthogonal field and C.H.E.F. (Contour-clamped Homogeneous Electric Fields)) into a series of 13 bands ranging from 0.1 Mb to over 2 Mb in size. Since this organism has only seven chromosomes (estimated to be 1-10 Mb), and ~90 copies of an 88-kilobase linear ribosomal DNA molecule (14% of genome), it was apparent that not all of these bands were whole chromosomes. However these bands were reproducibly obtained with the cell preparation used. They fell into three categories: i) four large poorly resolved DNA molecules (~2 Mb in size) which represent very large fragments or intact chromosomes, ii) eight faint bands ranging from 0.1 Mb to 2 Mb, iii) a prominent band in the apparent size range of about 0.15 Mb. Cloned Fragment V of an EcoRI digest of the ribosomal DNA, hybridized to the 0.15 Mb band indicating it contained the linear ribosomal DNA.

This chromosomal banding pattern was used to examine the stability and location of vector DNA in 16 transformed strains of *D. discoideum*. Each transformed strain was initially selected on the basis of G418 resistance with an integrating vector containing pBR322 sequences. Eleven transformants still carried pBR322 sequences after more than 60 generations of growth without selection on G418. All four strains transformed with constructs containing regions of the *D. discoideum* plasmid Ddpl had lost their pBR322 insert, indicating that integration of *Dictyostelium* plasmid DNA into chromosomes leads to instability. Orthogonal field electrophoresis of the eleven strains still carrying pBR322 sequences revealed at least seven different integrating sites for the transforming DNA. We conclude that these vectors have many possible sites of integration in the *D. discoideum* genome.

INTRODUCTION

In recent years the technique of Pulse Field Gel Electrophoresis has been developed to separate large DNA molecules up to several megabases in size (1-3). In the original apparatus, alternating pulses of electricity from two perpendicular electrode arrangements were used to achieve separation of DNA molecules up to 2 megabases (4). Subsequently, numerous electrode orientations and running conditions have been developed (5-7), and separation up to the 5-10 megabase size range has been reported (8,9,10). The genomes of...
several simple eukaryotes whose chromosomes span the range from several hundred kilobases to several megabases have been analysed using this technique (11-14). For organisms such as the yeast *Saccharomyces cerevisiae*, protozoa in the genera *Trypanosoma*, *Plasmodium* and *Leishmania*, or the dimorphic fungus *Candida albicans*, which are difficult to analyse cytologically, Pulsed Field Gel Electrophoresis represents the only convenient way to visualise their chromosome complement (4,15,16,17).

The cellular slime mold *Dictyostelium discoideum* is a simple amoeboid eukaryote that has been extensively studied as a model for cell differentiation (18). Genes for a number of interesting developmental and cytoskeletal proteins have been cloned (18). Reliable transformation is available using integrating vectors (19), and integration of transforming DNA by homologous recombination has been reported (20).

Classical genetic analysis of *D. discoideum* includes cytological studies of its 7 chromosomes in haploid strains (21), as well as the preparation of a genetic map, containing over 100 genes mapped to a particular chromosome (22). This organism has a total genome size of 55 megabases (23), about 8 megabases of which is in the form of ~90 copies of a 88 kilobase, linear ribosomal DNA molecule (24). This leaves an average size for each chromosome of ~7 megabases. We report here the use of pulse field gel electrophoresis separation techniques to analyse the *D. discoideum* chromosomes. Under the conditions used, subchromosomal fragments are reproducibly obtained, without the use of restriction enzymes (25,26). It is demonstrated that *D. discoideum* amoebae transformed with a pBR322-based vector integrate this foreign DNA at different chromosomal sites.

**MATERIALS AND METHODS**

**Strains and growth of cells**

Fifteen strains of *D. discoideum* transformed with integrating vectors based on plasmid B10 (19) were sent by Drs. Ahern and Firtel on silica gel from San Diego to Sydney where they were reactivated on *K. aerogenes* lawns on SM agar plates in the absence of G418. Amoebae from single colonies were prepared for silica gel and grown axenically for analysis by orthogonal field electrophoresis. Haploid *D. discoideum* strain AX3K (27), AX2 (28) and transformed derivatives were grown to stationary phase (~10⁷/ml) in axenic medium (28) in the absence of G418. This procedure, which involved at least 40 generations of growth, was carried out in the absence of G418 selection and in view of possible loss of transforming DNA, the strains were renamed.
Strains tested, with original name from the Firtel laboratory in brackets, were HU2457 (8P), HU2441 (B1OSXP), HU2454 (11Cl) all transformed with B1OSX; HU2456 (KGA9P), HU2461 (BP) both transformed with KGA9, a B10 based plasmid containing 2.5 kb of Discoidin coding and promoter sequences; HU2442 (p5-S), transformed with P5-S, a B10 based vector containing K5 cDNA; HU2444 (p5+S) transformed with P5+S, a B10 based vector containing K5 cDNA; HU2453 (GP) transformed with pSH7, a B10 based plasmid containing 1.7 kb of Discoidin coding and promoter sequences; strains B10 (not renamed), 2PG95S1, 2PG95S3 (renamed S1 and S3 respectively) all of which were transformed with B10. Finally four strains were transformed with vectors based on B10 but which carried sequences coding for the d3, d5 and part of the gl transcript from the endogenous D. discoideum plasmid Ddpl. The vector DNA did not replicate autonomously, but integrated into the chromosomes. These strains are HU2455 (EP), HU2458 (EC1) both transformed with CISS; HU2443 (DP) transformed with CISR2; HU2459 (IP) transformed with CIBB. Strain 1014 was obtained by transforming AX2 with B1OSX (Dingermann pers. commun.). While transformants were selected on the basis of G418 resistance (19), only strain 1014 showed a level of resistance to G418 substantially greater than that observed with the untransformed strains AX3K and AX2. Hence it is possible that in most of the transformants examined, the vector DNA had rearranged during cloning on SM agar and passaging in axenic medium free of the selective agent G418.

Saccharomyces cerevisiae strain AH22 (a, his'4, leu 2, can-1) was grown to a final concentration of $2 \times 10^8$/ml in yeast peptone dextrose (YPD) medium (9). Preparation of D. discoideum amoebae and S. cerevisiae for pulse field gel electrophoresis

Stationary phase D. discoideum amoebae were centrifuged (200 g for 3 min) and washed twice in sterile salt solution (10 mM NaCl, 10 mM KCl, 3 mM CaCl$_2$). The final pellet (-1 ml consisting of -10$^9$ cells) was mixed with an equal volume of 2X Seaplaque, low gelling temperature agarose in salt solution at 40°C. The resulting suspension was poured into an insert mold with the dimensions 4 cm x 0.8 cm x 0.3 cm and allowed to set at room temperature for 5 min. With a sterile scalpel blade the insert was cut into 16 plugs (1 cm x 0.4 cm x 0.15 cm) giving a final concentration of -5 x 10$^7$ cells/plug. The plugs were incubated in 10 ml of lysis buffer (0.01 M Tris, 1% Sarkosyl, 0.5M EDTA, pH 9.5, 1 mg/ml Proteinase K (Boehringer Mannheim)) at 50°C for 18–40 h, then stored in the lysis buffer at 4°C. The same chromosomal profiles were obtained when plugs were used immediately or stored for several months. Before running a gel each plug was incubated in 0.5 x TBE for 1 to 2 h (29).
S. cerevisiae was treated as described elsewhere (9), except that the final concentration of cells was $1.3 \times 10^9$/ml, rather than $2 \times 10^9$/ml. This gave $-8 \times 10^7$ cells/track.

**Equipment**

The gel tanks, the pulse field and orthogonal field electrode arrays were built according to the plans used by SPITHILL and GRUMONT (30). The C.H.E.F. (Contour-clamped Homogeneous Electric Fields) system was identical to that used by CHU, VOLLRATH and DAVIS (31), except that the resistors across the unclamped electrodes were attached to the electrode array, not the switching relay.

**Electrophoresis Conditions**

All systems were run in 0.5 x TBE (29). For the pulse field and orthogonal field electrode arrays, the optimal conditions for separation were 0.7% agarose, 15°C, 20 sec pulse time in both directions for 20 h. The voltage ratio for the pulse field system was 4 volts/cm E-W (uniform field), 10 volts/cm N-S (non-uniform field), after applying a constant 10 volts/cm in the N-S direction for 1 h to allow the DNA to enter the gel. Ten volts/cm were used in both directions for the orthogonal field system. The C.H.E.F. system was run at 2°C using 1% agarose 10 volts/cm, 45 sec pulses for 24 h. All gels were stained with ethidium bromide (0.8 ug/ml) for 1 h, then destained for 10 min before photographing under U.V. illumination.

**Southerns and Hybridizations**

All gels were transferred to zeta probe (Biorad), according to the protocol of REED and MAHN (32), with the addition of a depurination step, (0.25 M HCl, 30 min) to facilitate the transfer of large fragments of DNA.

Filters were hybridized at 68°C overnight in hybridization medium (33), containing $2 \times 10^8$ dpm/ug of nick-translated plasmid pBR322 as probe (34). The vectors used to transform D. discoideum carried sequences from this plasmid (19). After hybridization, filters were washed three times (2 x SSC, 0.5% SDS; 2 x SSC, 0.1% SDS; 0.1 x SSC, 0.1% SDS) at room temperature for 15 min. Ribosomal DNA was probed using cloned EcoRI fragment V DNA, obtained from R. Parish. In addition to the above washes these filters were subjected to a high stringency wash at 60°C for 3 h with 0.1 x SSC, 0.1% SDS. Autoradiography was carried out on Fuji X-ray film at -80°C for 2 - 4 days.

**RESULTS**

**Molecular karyotype of D. discoideum**

Since the chromosomes of D. discoideum are somewhat larger than those
Figure 1. The separation of chromosomes from *D. discoideum* strain AX3K and *S. cerevisiae* strain AH22 under three different pulse field gel electrophoresis systems. Tracks 1, 3, 5, *D. discoideum*, tracks 2 and 4, *S. cerevisiae*. Note that conditions used to separate *D. discoideum* chromosomes are suboptimal for resolving chromosomes of *S. cerevisiae*. Track 1: pulse field electrophoresis; tracks 2 and 3: C.H.E.F.; tracks 4 and 5: orthogonal field electrophoresis. Gels were run as described in the MATERIALS and METHODS. The bands are numbered for *D. discoideum* in the orthogonal field system, and the ribosomal DNA band (3) is also indicated on tracks 1 and 3.

Currently resolved by various pulse field electrophoresis systems, three different systems have been examined in order to optimise the conditions for separating *D. discoideum* chromosomal DNA. We have found that the non-uniform fields of both the pulse field (Figure 1, track 1) and orthogonal field (Figure 1, track 5) electrode arrays gave a sharper band clarity than the CHEF system (Figure 1, track 3). When attempting to resolve the small bands 4-10 (Figure 1), such band clarity becomes critical. Therefore, these two electrode arrays were adopted as the most useful systems for producing distinct, sharp bands.

A range of different conditions were tried before the best parameters for separation were found. For these systems the following conditions were tried; gel concentrations 0.2% to 2%, temperatures 2°C to 37°C, pulse times 14 sec to 700 sec, run lengths 8 h to 170 h and voltages 5 volt/cm to 15 volt/cm.

The pulse field electrophoresis system produced an S-shaped banding pattern with only a slight widening of the bands at the bottom of the gel.
All DNA that entered the gel under this system could be resolved, although the pulse regime for optimal separation was very narrow (1-2 sec around a 20 sec pulse). This meant that all other parameters (gel concentration, buffer depth, temperature, voltage ratio and voltage strength) had to be set very accurately to achieve optimal separation. In practice it was difficult to achieve reproducible gel patterns with this system. On the other hand the pattern achieved with the orthogonal field system was much more robust.

As shown in Figure 1 (tracks 1 and 5) the basic banding patterns of the two systems were similar. The only significant difference was in the high molecular weight region at the top of each gel. Our best runs have resolved this region into four bands in the pulse field system and three bands in the orthogonal field system. We are currently working to improve separation in this region with C.H.E.F. (see 10). Bands 3, 12 and 13 were consistently more strongly stained than the other bands. Since there are only seven chromosomes in haploid *D. discoideum* we concluded that many of the bands observed represented some type of discrete chromosomal breakage that was reproducible using the preparation procedure described. The faintly stained bands (1, 2, 4-11) were always of similar intensity and could be increased or reduced by varying the lysis conditions. If the cells were heat shocked at 90°C for 3 minutes prior to taking them through the lysis procedure all the faintly staining bands were reduced. Similarly by washing the cells in sterile salt solution at pH 11, then incubating them in lysis buffer at the same pH, these bands were almost eliminated. These results are consistent with bands 1, 2, 4-11 arising as a result of specific enzymic digestion of chromosomal DNA.

**Size of *D. discoideum* bands**

The smallest band of *S. cerevisiae* ran slightly behind *D. discoideum* band 7 in the orthogonal field system (Figure 1, tracks 4 & 5). The smallest yeast chromosome is known to be 0.2 Mb (35), so *D. discoideum* bands 1-7 ranged in size from ~0.1-0.2 Mb. Bands 8-10 ranged from 0.2--2 Mb, while bands 11-13 ran in a region of the gel which did not resolve well but where the largest yeast chromosomes were found.

The largest yeast chromosome (chromosome XII) has been estimated to be in the size range of 2.2 Mb to 2.5 Mb (35). To separate DNA molecules in this size range has required us to operate at the limits of the orthogonal and C.H.E.F. systems (8,10). This has required low voltages and subsequently long run lengths which have not yet been very successful. However, at long pulse times at least one of the bands in this region is significantly heavier than
Figure 2. A. Ethidium bromide stained orthogonal field electrophoresis gel of (track 1) equine herpesvirus type 1 DNA and (track 2) *D. discoideum* AX3K amoebae. Numbers refer to bands described in Figure 1. B. Hybridization of cloned ribosomal DNA EcoRI fragment V to AX3K DNA of *D. discoideum* separated in the orthogonal field system. Track 1, ethidium bromide stained gel, Track 2, autoradiograph after probing with fragment V.

the largest yeast chromosome. It should be noted that some DNA remains in the well even under long pulse times.

Consistently the most prominent amongst the smaller bands was band 3. Some of our gels show a faint band which runs slightly ahead of this band. Since this band is usually concealed by band 3 we have not labeled it. Band 3 ran at a position comparable to linear double stranded equine herpes virus type 1 DNA (Figure 2A) which has been accurately sized by restriction mapping at 0.155 Mb (36). Figure 2B shows that band 3 contains DNA that hybridises to a cloned EcoRI fragment of the *D. discoideum* ribosomal DNA palindrome. Essentially no hybridization was observed with any other band, although there was a strong signal in the loading well. Similar retention of DNA in the wells has also been reported in Trypanosomes (11,17), *Plasmodium* (12,37) and Leishmania (38).

Mapping of bacterial plasmid pBR322 DNA in *D. discoideum* transformants

In Figures 1 and 2 it was shown that *D. discoideum* chromosomal material
Figure 3. A, C, Ethidium bromide staining and B, D autoradiography of DNA from *D. discoideum* transformed with a pBR322-construct then separated on the orthogonal field system. In both gels track 6 (AX3K) is the negative control, while track 1 (1014) is a positive control. HU2442 (B, track 5), HU2459 (D, track 2) and HU2443 (D, track 5) show no hybridization, indicating that these transformants had lost their pBR322-construct. The other strains carry pBR322 DNA integrated into bands as indicated in B and D.
was fractionated into 13 bands on an orthogonal field system. Band 3 contained the ribosomal DNA palindrome, bands 12 and 13 contained small intact chromosomes or large chromosomal fragments at least 2 Mb in size, while bands 1, 2, 4-11 represented possible chromosome fragments. The fact that banding was reproducible suggested that it might be possible to map genes to particular bands. We have demonstrated this by assigning pBR322 sequences to various bands in strains transformed by integrating vectors which contain sequences from this plasmid.

Of the 16 independent transformants we studied, 11 retained pBR322 sequences after 60 generations of growth in the absence of G418 selection. All four strains tested (HU2443, HU2459, HU2455, HU2458), which had been transformed with vectors based on B10SX but which carried in addition a segment of endogenous \textit{D. discoideum} plasmid Ddpl, lost their pBR322 sequences. Eleven of 12 strains transformed with B10SX or related plasmids retained pBR322 sequences in the absence of selection. Figure 3 shows the results obtained for a representative group of transformants.

Lanes 1-5 of both gels in Figure 3 represent 9 different transformation events; transformant 1014 was run in both gels as a positive control. Strain AX3K, which was the recipient strain for transforming DNA, was used as a negative control. Plasmid pBR322 did not hybridize to any AX3K DNA band in the gel or in the wells (Figure 3B, 3D track 6). Transformants HU2442, HU2459 and HU2443 (Figure 3B track 5; 3D tracks 2, 5) likewise showed no hybridization in either the gel or the wells after 60 generations. A very strong signal in bands 12-13 was always observed with 1014 (Figure 3B, 3D track 1), indicating multiple insertions of this vector sequence in the same region. The remaining 5 transformants in Figure 3 showed hybridization of pBR322 to specific bands; (HU2457, band 7; HU2453, bands 10, 11, 12-13; S1 and S3, bands 6 and 7; strain B10 hybridized strongly to the well). The strong signal obtained with B10 suggests that the vector integrated into chromosomal material that was too large to leave the well under the pulse conditions used. Five further transformants contained pBR322 sequences integrated into band 3 (HU2454), band 12 & 13 (HU2444), bands 11-13 (HU2441), several bands (HU2461) and the well (HU2456) (data not shown). Hence most of the 11 strains containing pBR322 sequences showed a different pattern of integration into the chromosomes.

\textbf{DISCUSSION}

By the use of pulse field gel electrophoresis we have demonstrated the separation of at least 13 discrete bands using the standard protocol for
producing intact naked DNA (8). Most of these bands are chromosomal fragments ranging in size from 0.1 Mb to 2 Mb. However at least two chromosome-sized bands were produced. These could represent chromosomes that do not fragment, as the transformant 1014 labels only these large bands at the top of the gel. The smaller bands are faint, but reproducible and can be reduced by a brief heat treatment of the cells, or by raising the pH of the lysis buffer to 11. If nuclei are prepared prior to DNA preparation, these bands are also eliminated. This could point to a specific enzymatic activity associated with the cytoplasm.

Evidence from the hybridization of the ribosomal EcoRI fragment V probe, suggests that the prominent band 3 (Figure 1) is ribosomal DNA. However, when the size of this band was compared with herpesvirus type 1 and S. cerevisiae chromosomes, it was located at 0.15 Mb, nearly twice its predicted size (88-kilobase). The evidence that ribosomal DNA exists as a 88-kilobase palindromic dimer, which is extrachromosomal (24) is substantial, including visualisation of the linear molecule by electron microscopy (39). Anomalous migration of relatively 'small' pieces of DNA is not without precedent in these systems as circular plasmids have been shown to run in a position expected of DNA much greater than their actual size in these gels (40,41). Certainly the ribosomal DNA band of D. discoideum has always been found in the same position in all three pulse field systems used.

In the very large region (2 Mb or greater), separation has proven very difficult and, as yet, it is unclear how many bands are present. Under optimal conditions each system has shown at least 3 and possibly 4 bands in this region although the resolution of these bands is poor. We are still optimising the conditions with the orthogonal and C.H.E.F. systems, which have the separation potential for 5-10 Mb (8,10).

The bands already produced however, have proved invaluable in mapping the positions of insertion of a pBR322-based vector into the D. discoideum genome. Constructs containing pBR322 integrate into the chromosomes at a number of different sites. In some cases only one band is labeled (e.g. HU2457, HU2454) and other times several bands are labeled (e.g. HU2453, HU2461). Whether this indicates that in these transformants the vector inserted at several sites, or that the chromosomes fragmented in several steps and the bands represent different sized fragments of the same chromosome, remains to be established by classical genetic analysis of these transformants. If part of the D. discoideum plasmid Ddpl is included in the vector the transforming DNA is unstable and is lost by 40-60 generations in non-selective media.
Apart from screening for chromosomal integration of transformants the use of chromosomal fragments opens the way for correlating each band with a specific cytological chromosome and a genetic linkage group. This will facilitate classical genetic analysis, such as mapping of chromosome rearrangements, as well as providing a quick means for mapping any gene for which a cloned probe is available.

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