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Identification of regions essential for extrachromosomal replication and maintenance of an endogenous plasmid in *Dictyostelium*

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ABSTRACT

Initial experiments with the endogenous 12.3 kb *Dictyostelium discoideum* plasmid Ddp1 led to the generation of a large shuttle vector, Ddp1-20. In addition to Ddp1, this vector contains pBR322 and a gene fusion that confers G418 resistance in *Dictyostelium* cells. We have shown that Ddp1-20 replicates extrachromosomally in *Dictyostelium* cells and can be grown in *Escherichia coli* cells (1). We have now examined deletions within this vector to identify the elements essential for extrachromosomal replication and stable maintenance of the plasmid. We find that a 2.2 kb fragment is sufficient to confer stable, extrachromosomal replication with a reduction in copy number from about 40 to ~10-15 copies per cell. Vectors containing additional Ddp1 sequences have a higher copy number. The 2.2 kb region contains none of the complete, previously identified transcription units on Ddp1 expressed during vegetative growth or development. These results suggest that gene products expressed by Ddp1 are not essential for replication, stability, or partitioning of the plasmid between daughter cells. Vectors carrying only the 2.2 kb fragment plus the gene fusion conferring G418 resistance transform *Dictyostelium* cells with high efficiency using either calcium phosphate mediated transformation or electroporation. Finally, we have examined the relative levels of expression of actin promoters driving neo^R genes when in extrachromosomal or integrating vectors.

INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* has been used for studies of basic, eukaryotic processes such as development, chemotaxis, signal transduction, adhesion, motility and gene regulation (2-9). Many developmentally regulated genes have been isolated and characterized. This has led to an understanding of some of the physiological signals that regulate the expression of these genes and has helped refine the theories of how development is controlled in this organism. The development of transformation protocols and shuttle vectors for stable introduction of DNA sequences into *Dictyostelium* has led the way to an analysis, at the sequence level, of the molecular mechanisms regulating these genes (1,10-18). Expression of altered gene products by expression of antisense RNAs or by gene disruption (19-23; Rubino, Mann, Hori, Pinko and Firtel, manus. in prep.) has allowed the dissection of specific cellular and developmental processes.

Endogenous plasmids have been identified for only a few eukaryotic organisms. The best characterized of these is the 2 μ circle from yeast (24). Extrachromosomal nuclear plasmids have been isolated from several strains of *Dictyostelium discoideum* (25,26; Firtel, unpub. obser.). Of these plasmids, Ddp1 has been examined in greatest detail (1,25-27) and transcription units expressed during growth and development have been mapped (25). Ddp1 was cloned into pBR322 (1). A shuttle vector carrying selectable markers for both *Dictyostelium* and *E. coli* was generated by recombination, *in vivo*, of the pBR322 Ddp1 clone with vector B10S, a pBR322 based plasmid carrying an Actin 6-neo^R gene fusion that confers G418 resistance in *Dictyostelium* (11). In *Dictyostelium* cells transformed with B10S, the vector is stably integrated into the chromosome in a tandem array (1,10,11). The resulting recombinant vector, Ddp1-20, carries ampicillin resistance and the pBR322 origin for

growth and selection in *E. coli* and G418 resistance for selection of transformants in *Dictyostelium*. This vector is stably maintained in the presence or absence of selection in *Dictyostelium*. It replicates extrachromosomally and thus presumably contains a functional origin of replication from Ddp1 (1). We are interested in identifying the sequences essential for extrachromosomal replication and stable maintenance and to identify possible transcription units on the plasmid that may be required for these functions. In addition, Ddp1-20, due to its large size (~19 kb), has limited usefulness as a shuttle vector for the expression of genes in *Dictyostelium*. Elimination of nonessential sequences would allow the construction of vectors with greater utility. We have made deletions in Ddp1-20 and assayed their effects on extrachromosomal replication and stable maintenance in *Dictyostelium*. A ~2.2 kb fragment has been shown to carry sufficient information for extrachromosomal replication and maintenance. Using a recently established protocol for electroporating DNA into *Dictyostelium* (29), we show that extrachromosomal vectors introduced in this manner can be used to transform DNA at a relatively high frequency. Relative levels of expression of fusion genes on these vectors have been examined.

MATERIALS AND METHODS

Growth and transformation of *Dictyostelium* cells

Strain KAx-3 was grown axenically, and transformants were selected for G418 resistance using the procedures of calcium phosphate or electroporation as previously described (10,28,29).

Isolation and Analysis of RNA and DNA from Dictyostelium Cells

RNA was isolated at specific times as previously described (18,30). RNA was sized on denaturing gels, blotted, and probed (18,30).

Whole-cell *Dictyostelium* nucleic acids were isolated from axenically grown vegetative cells. DNA, either undigested or digested with restriction endonucleases, was sized on 0.65% agarose gels, blotted, and probed as described previously (1,10,28). To determine the number of vector copies per haploid genome, purified linearized plasmid DNA in amounts equivalent to 1, 5, 20, 100, and 200 copies of plasmid per haploid equivalent of *Dictyostelium* genomic DNA was added to a known amount of CsCl purified DNA from untransformed *Dictyostelium* cells in adjacent lanes with DNA from the transformants. Because whole cell nucleic acid preparations were used to analyze DNA from transformants, it was not possible to directly determine the amount of transformant DNA loaded in each lane. Instead, we ran fractions of an equivalent aliquot of DNA digested with *Eco*RI. The gel was blotted and probed with a cloned fragment of *Dictyostelium* rDNA (Band IV), which is present at 180 copies per cell (31,32). The intensity of the hybridization to Band IV DNA relative to that seen with a known quantity of CsCl purified DNA is used to quantitate the DNA loaded in each well.

To quantitate the relative levels of hybridization, autoradiograms were scanned using a Hoefer GS-300 scanning apparatus and the average band intensity was determined using the Hoefer GS-370 software on a Macintosh II computer.

Vector descriptions

Vectors used in these experiments (see Figure 1) were constructed from previously described plasmids. Cartoons showing the structure of the vectors used in this manuscript are given in Figure 1. Ddp1-20 (1), pA15TX (13), and B10SX (11) have been described previously.

Ddp1-20 (1) contains the complete sequence of the *Dictyostelium* nuclear plasmid Ddp1 cloned into the *Sph*I site of pBR322 and a 2.2 kb Actin 6-neo^R-Actin 8 terminator gene fusion fragment for G418 resistance (10,11). For simplicity, we have divided Ddp1-20 into nine cassettes bounded by restriction enzyme recognition sites. Descriptions that follow run left to right in Figure 1. Cassette A is a 1.2 kb fragment between the unique *Sal*I in pBR322 and the leftward most *Kpn*I site of the molecule. This fragment spans the boundary between

pBR322 sequences and Ddp1 sequences. Cassette B spans across the two *Kpn*I fragments. Cassette C runs from the right most *Kpn*I site to the next HindIII site. Cassettes D and E are the next 500 bp and 2.2 kb *Hin*dIII fragments respectively. Cassette F is the 800 bp *Bgl*II bounded fragment. Cassette G is the 3.2 kb *Bgl*II-BamHI fragment. The BamHI site represents the boundary between the end of the Ddp1 sequences and the 3' end of the Actin 6-neo^R fusion. Cassette H is the Actin 6-neo^R fusion described above. Cassette I represents bacterial vector sequences. pBR322 is the bacterial vector in Ddp1-20. Cassette A contains vector sequences between the *Sal*I and *Sph*I sites of pBR322 as well as Ddp1 sequences. Cassettes B, C, D, E, F, and G contain only Ddp1 sequences. The linear arrangement of cassettes left to right is ABCDEFGHI.

CI was derived from Ddp1-20 by removing the two *Kpn*I fragments (Cassette B) and the 800 bp *Bgl*II fragment (Cassette F). It contains the Ddp1-containing Cassettes A, C, D, E, and G, the Actin 6-neo^R Cassette H and pBR322 vector sequences all from Ddp1-20. It is equivalent to Ddp1-20 with Cassettes B and F removed. The linear arrangement of cassettes is ACDEGHI.

CIKS was derived from CI by removing the 1.2 kb *Kpn*I-*Sal*I fragment (Cassette A). It contains the Ddp1-containing Cassettes C, D, E, and G, the Actin 6-neo^R Cassette H, and pBR322 vector sequences. It is equivalent to Ddp1-20 with Cassettes A, B, and F deleted. The linear arrangement of cassettes is CDEGHI.

VIIBB contains the 3.2 kb *Bgl*II-*Bam*HI fragment of Ddp1-20 (Cassette G) cloned into the *Bam*HI site of B10SX. B10SX has bacterial vector sequences derived from pUC9 (11). Cassette G represents the only sequences from Ddp1 contained in VIBB. The linear arrangement of cassettes is GHI.

CIBB was made from CI by deleting the 3.2 kb *Bgl*II-*Bam*HI fragment (Cassette G). It consists of the Ddp1-containing Cassettes A, C, D, E, the Actin 6-neo^R Cassette H, and pBR322 vector sequences. It is equivalent to Ddp1-20 with Cassettes B, F, and G deleted. The linear arrangement of cassettes is ACDEHI.

CIKSBB was made from CIKS by deleting the 3.2 kb *Bgl*II-*Bam*HI fragment (Cassette G). It consists of the Ddp1-containing Cassettes C, D, and E, the Actin 6-neo^R Cassette H, and pBR322 vector sequences. It is equivalent to Ddp1-20 with Cassettes A, B, F, and G deleted. The linear arrangement of cassettes is CDEHI.

A15E-A6 contains the 4.2 kb fragment between the right most *Kpn*I site of Ddp1-20 and the left most *Bgl*II site (Cassettes C, D, and E) cloned into the bacterial vector pAT153. In addition, A15E-A6 contains the Actin 15-NPTI (neo^R) gene fusion from pA15TX (12,13) rather than the Actin 6-neo^R gene (NPTII) fusion (Cassette H) for G418 resistance. It also contains an Actin 6 promoter-Actin 8 termination construct (Cassette J) made from the Actin 6-neo^R gene fusion from B10SX that can be used for expressing genes under the control of the Actin 6 promoter. The modified Actin 6-neo^R fusion was made by deleting the 900 bp *Pst*I fragment in the neo^R gene and replacing it with an *Xba*I linker, thus deleting the majority of the NPTII coding sequences. Cassettes C, D, and E are the only Ddp1 derived sequences in the plasmid. The linear arrangement of cassettes is HJEDCI.

VIE11 contains the 4.2 kb fragment between the right most *Kpn*I site of Ddp1-20 and the left most *Bgl*II site (Cassettes C, D, and E) cloned into Vector I (see below), a pBR322-based vector containing the Actin 6-neo^R fusion (Cassette H) and the DiscI-α promoter and the terminator from the 3' end of the 2-H3 prespore gene (19) (Cassette K). Cassettes C, D, and E are the only Ddp1 derived sequences in the plasmid. The linear arrangement of cassettes is HCDEKI. Cassette K was constructed to express RNAs from the DiscI-α promoter.

7AH3 was made by inserting the 2.2 kb *Hin*dIII fragment of Ddp1 (Cassette E) into the *Hin*dIII site of 7A, a pUC19 vector with the Actin 6-neo^R fusion gene (Cassette H) that also contains a shortened DiscI-α promoter-2-H3 fusion (Cassette K). Cassette E represents the only Ddp1 derived sequences in the plasmid. The linear arrangement of cassettes is EKHI.

CIKSBBH was made from CIKSBB by deleting the 500 bp (Cassette D) and 2.2 kb (Cassette E) *Hin*dIII

fragments of CIKSBB. The only Ddp1-derived fragment it contains is Cassette C as well as the Actin 6-neo^R Cassette H and pBR322 vector sequences. It is equivalent to Ddp1-20 with Cassettes A, B, D, E, F, and G deleted. The linear arrangement is CHI.

ZD5AN contains the 500 bp *Hin*dIII fragment of Ddp1 (Cassette D) cloned in a pGEM3-Z bacterial vector (Promega Biotech) with the Actin 6-neo^R fusion (Cassette H). Cassette D represents the only Ddp1-derived sequences in the plasmid. The linear arrangement of cassettes is HDI.

15KA contains the 7.4 kb fragment between the unique *Kpn*I site and the unique *Bam*HI site (Cassettes C, D, E, F, and G) of CI in a pBR322 vector with the Actin 6-neo^R fusion (Cassette H). It contains Cassettes C, D, E, F, and G derived from Ddp1. In addition, it contains sequences for expressing genes under the control of the Actin 15 promoter (Cassette L) isolated from pA15TX (13). The linear arrangement of cassettes is LCDEGHI.

B10SX (11) contains the Actin 6-neo^R fusion cloned in a pUC9 backbone. It contains no Ddp1-derived sequences and will not replicate extrachromosomally. The linear arrangement of cassettes is HI.

Vector I contains the Actin 6-neo^R fusion cloned in a pBR322 backbone. It also contains a DiscI-α-2-H3 fusion (Cassette K) for expression of genes under the control of the DiscI-α promoter. This fusion has been described previously (19) and contains the *Bgl*II-*Kpn*I fragment from the DiscI-α gene (33,34) fused to a *Kpn*I-*Eco*RI fragment carrying the 3' end and terminator of the prespore gene 2-H3 into the *Bam*HI-*Eco*RI sites of pBR322.

RESULTS

Construction of deletion series

Figure 1 shows the parental plasmid, Ddp1-20. It was isolated from *Dictyostelium* transformants that had been cotransformed with vector B10S (carrying a gene fusion conferring G418 resistance cloned into pBR322) and plasmid pBMW3 [composed of Ddp1 cloned into pBR322 at the unique *Sph*I site in both DNAs (1)]. A plasmid with the structure of Ddp1-20 was repeatedly isolated from independently transformed, G418 resistant populations of *Dictyostelium* cells. Mapping data indicated that Ddp1-20 appears to have been formed by recombination between the homologous pBR322 sequences of pBMW3 and B10S. Ddp1-20 transforms *Dictyostelium* at high frequency and is stably maintained as an extrachromosomal vector in *Dictyostelium* cells even in the absence of G418 selection (1).

To identify the regions required for extrachromosomal replication, we made a number of vectors containing deletions of sequences within Ddp1. These are also shown in Figure 1. All of the plasmids are in a pBR322 or pUC based backbone and still contain the Actin 6-neo^R gene fusion from B10S encoding G418 resistance in *Dictyostelium*. Thus, all the vectors were expected to be able to yield stable transformants, either by integrating or by replicating extrachromosomally. It should be mentioned that we attempted to clone a number of the larger constructs into pUC derived vectors because these vectors produce higher yields of DNA in *E. coli* (35). Vector 7AH3, which contains only a 2.2 kb *Hin*dIII fragment from Ddp1 is stable in pUC vectors, while the attempts to clone many of the larger fragments often resulted in rearrangements.

Analysis of vector DNA in transformants

The DNAs shown in Figure 1 were transformed into *Dictyostelium* strain KAx-3 using the calcium phosphate procedure and stable transformed cell lines capable of growing in the presence of G418 were selected. Strain KAx-3 lacks the endogenous Ddp1 plasmid and does not contain any DNA sequences that cross-hybridize with Ddp1 (1). There is also no evidence for any other endogenous plasmid in this strain (1). To determine whether the DNA was integrated within the chromosomes or was replicating extrachromosomally and to deter-

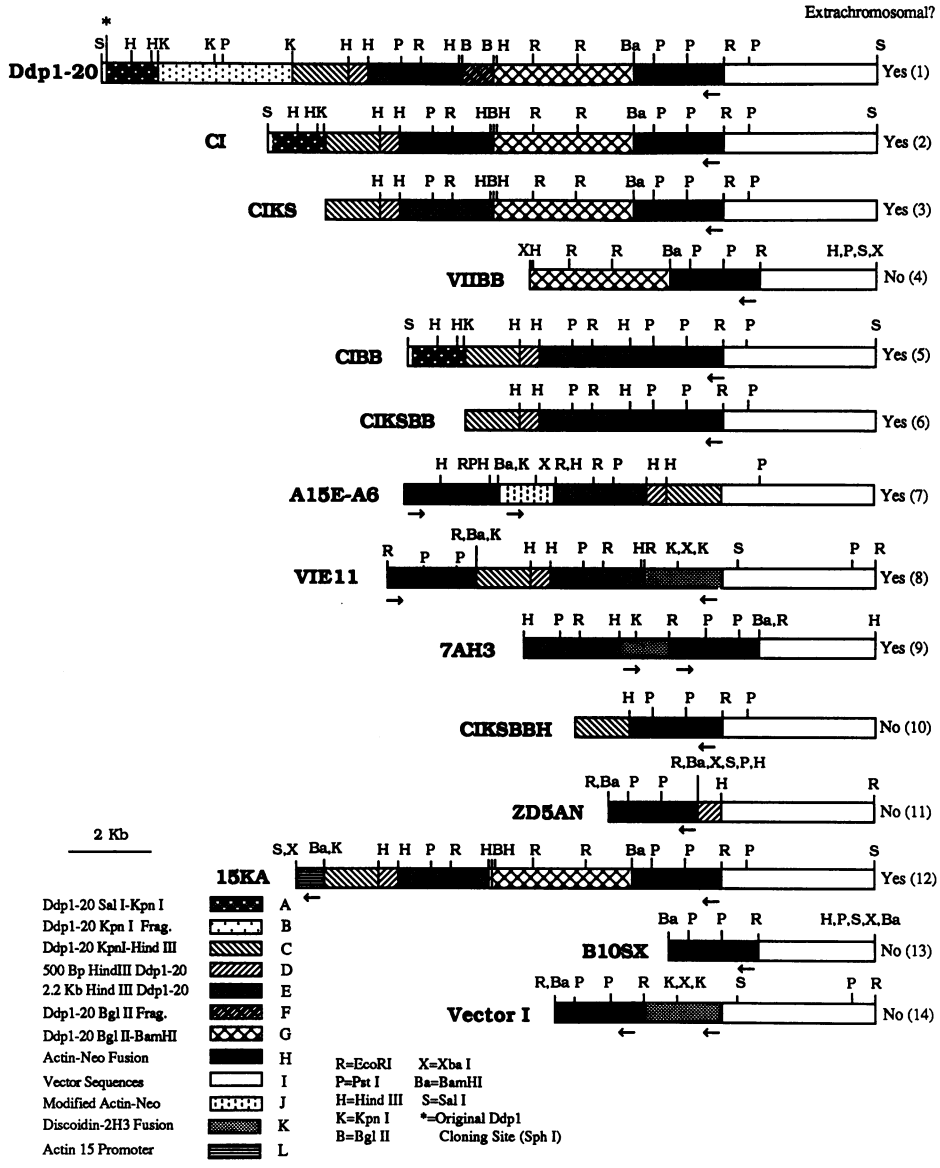

Figure 1. Restriction maps of the *Dictyostelium* transformation vectors used in these studies. All but A15E-A6 carry the Actin 6-neo^R gene fusion encoding resistance to G418. A15E-A6 carries the Actin 15-NPTI (neo^R) gene fusion from pA15TX (12,13). Ddp1-20 has been described previously (10). It contains the entire Ddp1 sequence cloned into the *SphI* site of pBR322. Constructs Ddp1-20, CI, CIKS, CIBB, CIKSBB, A15E-A6, VIE11, and 7AH3 replicate extrachromosomally; all others except 15KA are integrating vectors. 15KA is a vector used in the construction of vector A15-A6 and should replicate extrachromosomally. Full descriptions of each vector are given in the Materials and Methods. Each plasmid is numbered on the far right. These numbers are used to label the lanes in the gels in Figure 2 and indicate the vector used in making the transformants. The ability of a vector to replicate extrachromosomally is given in Figure 1 to the right of the map of each plasmid.

TABLE I

Vector	Vector copy number in transformants: Copies/haploid genome	Extrachromosomal
Ddp1-20	40	yes
CI	29	yes
CIKS	6	yes
CIBB	17	yes
CIKSBB	6	yes
7AH3	13	yes
B10SX	200	no
VIIBB	55	no
CIKSBBH	307	no

Vector copy number was determined as described in the Materials and Methods and Results sections. Copy numbers shown are from the data in Figures 2 and 3. Vector copy numbers for the extrachromosomal vectors vary by ±50%. These numbers are representative of the median values observed between experiments as well as the values observed in these experiments. Values for integrating vectors can vary between 10 and 400 copies per haploid genome (data not shown).

mine the approximate copy number of the vector within the cells, whole cell DNA was isolated from transformants (see Materials and Methods). Whole-cell DNA, either undigested or digested with a restriction enzyme that cuts once within the appropriate vector, was then size fractionated by electrophoresis on agarose gels. The DNA was blotted to membrane filters and hybridized with a pGEM4 nick-translated probe. We previously showed that vectors derived from B10S lacking Ddp1 sequences stably transform axenic strains, which lack the endogenous plasmid, at a frequency of ~50-200 colonies per plate of 10^7 cells (10,11,28). Analysis of DNA from the G418 resistant colonies indicates that the vector is present in the chromosomes in a tandem array of between 10-350 copies per cell depending on the vector. As has been previously described, the copy number is variable from one transformed clone to another (data not shown; Datta and Firtel, unpub. obser.; 10-12,28). These vectors are stably maintained whether the cells are grown in the presence or absence of G418 selection (10,11,28). Ddp1-20 is also stably maintained, but as an extrachromosomally replicating vector at a copy number of ~20-50 per cell (see Table I and ref. 1). DNA blot analysis of chromosomal DNA digested with an enzyme that cuts once within the integrated plasmid vector reveals a band with the same mobility as the linearized vector indicating the copies are in a tandem array, the intensity of which is dependent on the copy number of the integrating DNA. End fragments (juncture pieces between genomic DNA and the tandem insert) produced by this digestion are often not visible over the background, particularly in populations of transformants. Extrachromosomal DNA analyzed in the same manner also produces a linearized band, but no end fragments. If uncut, whole-cell DNA is sized on agarose gels, the integrated vector DNA comigrates with the bulk of the uncut chromosomal DNA. However, extrachromosomally replicating vector DNA migrates as either a nicked circle (predominant form seen with large vectors) or as a supercoil. Very little or no DNA migrates with the bulk of the chromosomal DNA for Ddp1-20 (1,10,11) (see further discussion below).

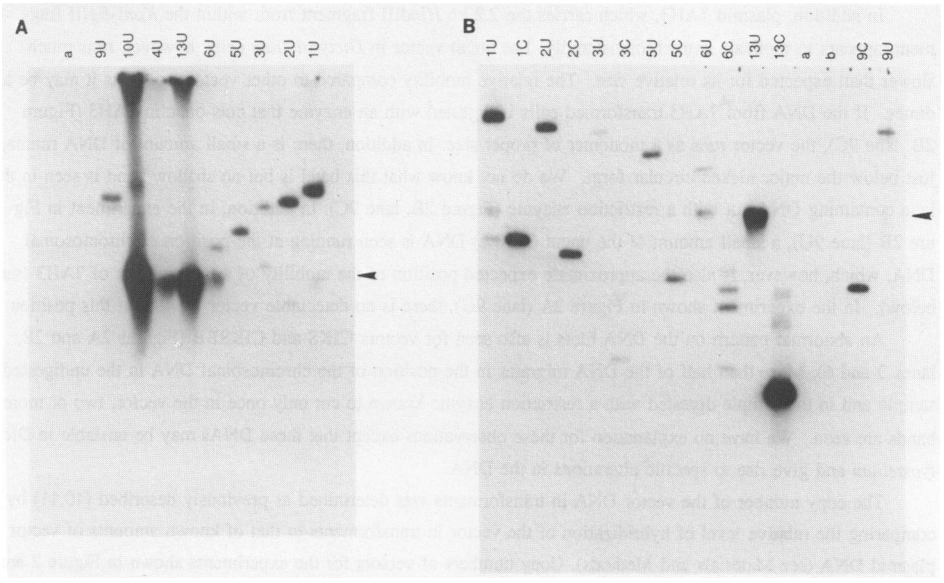

Figure 2. Analysis of *Dictyostelium* transformation vectors separated on a 0.65% agarose gel. Probe used in this blot was made from nick translated pGEM4 plasmid DNA, which is complementary to all the *E. coli* cloning vectors used. The arrow indicates the position of integrated uncut vector DNA.
Part A. The lanes contain DNA from transformants carrying the following vector DNAs (Note that the lanes are labeled according to the DNA vector used to transform the cells. The numbers correspond to the numbering of the plasmids given in Figure 1.): lane 1, Ddp1-20, lane 2, CI; lane 3, CIKS; lane 4, VIIBB; lane 5, CIBB; lane 6, CIKSBB; lane 9, 7AH3; lane 10, CIKSBBH; lane 13, B10SX; lane a, untransformed KAx-3 DNA. Note: the "U" on lane numbers indicates DNA is undigested with restriction endonucleases.
Part B. Analysis of *Dictyostelium* transformation vectors either digested or not digested with a restriction enzyme that cleaves the vector only once. Probe was made from nick-translated pGEM4 plasmid DNA. The arrow indicates the position of integrated uncut vector DNA. Lane designation is the same as in Part A. The "C" on lane numbers indicates the DNA is digested with a restriction enzyme that cuts once within the vector. The enzymes used are: 1C, *Sal*I; 2C, *Bam*HI; 3C, *Bam*HI; 5C, *Sal*I; 6C, *Eco*RV; 9C, *Eco*RV; 13C, *Bam*HI; b, control *Dictyostelium* DNA digested with *Eco*RI.

The results of the experiments using the vectors depicted in Figure 1 are shown in Figure 2. Quantitation of the copy number of some of these vectors is given in Table I. As can be seen, all vectors carrying the ~4.2 kb region between the *Bgl*II and adjacent *Kpn*I site of Ddp1 replicate extrachromosomally. On the other hand, vectors that do not carry the 4.2 kb *Kpn*I/*Bgl*II fragment integrate in a tandem array (see Figure 1 and vectors VIIBB, CIKSBBH, and B10SX in Figure 2), indicating that the 4.2 kb *Kpn*I-BglII fragment carries all the necessary sequences to replicate stably as extrachromosomal molecules. With certain extrachromosomally replicating vectors, some vector DNA is seen migrating at the position of uncut chromosomal DNA (specifically see lanes 1U and 6U in Figure 2). The relative amount of DNA migrating in this position is dependent on the vector and is variable from experiment to experiment; in some DNA preparations it is not seen at all. This will be addressed in more detail in the Discussion. Most of the vector DNA for extrachromosomally replicating plasmids migrates as nicked circles; however, some DNA has the approximate mobility of supercoiled forms of the vector (more rapidly migrating forms).

In addition, plasmid 7AH3, which carries the 2.2 kb *Hin*dIII fragment from within the *Kpn*I-*Bgl*II fragment, appears to replicate extrachromosomally. The uncut vector in *Dictyostelium* cells, however, runs much slower than expected for its relative size. The relative mobility compared to other vectors suggests it may be a dimer. If the DNA from 7AH3 transformed cells is digested with an enzyme that cuts once in 2AH3 (Figure 2B, lane 9C), the vector runs as a monomer of proper size. In addition, there is a small amount of DNA running just below the major nicked circular form. We do not know what this band is but no shadow band is seen in the lane containing DNA cut with a restriction enzyme (Figure 2B, lane 9C). In addition, in the experiment in Figure 2B (lane 9U), a small amount of the uncut (5-10%) DNA is seen running at the position of chromosomal DNA, which, however, is also the approximate expected position of the mobility of a linear dimer of 7AH3 (see below). In the experiment shown in Figure 2A (lane 9U), there is no detectable vector running at this position.

An abnormal pattern on the DNA blots is also seen for vectors CIKS and CIKSBB (Figures 2A and 2B, lanes 3 and 6). More than half of the DNA migrates in the position of the chromosomal DNA in the undigested sample and in the sample digested with a restriction enzyme known to cut only once in the vector, two or more bands are seen. We have no explanation for these observations except that these DNAs may be unstable in *Dictyostelium* and give rise to specific alterations in the DNA.

The copy number of the vector DNA in transformants was determined as previously described (10,11) by comparing the relative level of hybridization of the vector in transformants to that of known amounts of vector plasmid DNA (see Materials and Methods). Copy numbers of vectors for the experiments shown in Figure 2 are given in Table I. As can be seen, vectors carrying the entire Ddp1 plasmid or deletions removing the two *Kpn*I fragments (Cassette B) and the *Bgl*II-*Bgl*II fragment (Cassette F) have copy numbers of 20-50, similar to that seen previously with Ddp1-20 (1). Vector CIBB which has a deletion of the *Bgl*II-BamHI fragment (Cassette G) and vector 7AH3 which carries on the 2.2 kb *Hin*dIII fragment (Cassette E) have copy numbers of 10-20. Vectors CIKS and CIKSBB, which have further deletions from their parental vector (CI and CIBB respectively), have much lower copy numbers and have unusual banding patterns suggesting instability. Interestingly, VIE11 and A15E-A6, which use the Actin 15-NPTI (neo^R) gene to encode G418 resistance rather than the Actin 6-neo^R gene, have copy numbers of 10-20. They do not show an unusual banding pattern and thus do not appear to be unstable in *Dictyostelium*. VIE11 carries the same parts of Ddp1 as CIKSBB. It also carries a promoter and terminator for expressing other genes, which, in itself, does not appear to affect stability or copy number in other experiments we have done (data not shown). The major difference between the two vectors, CIKS and CIKSBB, that show the unusual pattern on gels, and the other vectors is the organization of the cassettes on the vector. Whether or not this is the important factor for causing the altered banding pattern is not known. From these data several conclusions can be made. First, the *Bgl*II-*Bgl*II and *Bgl*II-*Bam*HI fragments (Cassettes F and G) do not seem to be essential for plasmid copy number or affect mobility patterns. The *Bgl*II-*Kpn*I regions, Cassettes C, D, and E or Cassette E alone, is sufficient for a moderate copy number and stability (10-20 copies/genome). The presence of the *Sal*I-*Kpn*I fragment (Cassette A) appears to result in a higher copy number (compare CIBB and CIKSBB). The presence or absence of that sequence also correlates with the presence or absence of rearrangements for the CIKS or CI and CIKSBB or CIBB sets. Organization of the sequences may be important for stability in *Dictyostelium*.

Transformation efficiency of extrachromosomal vectors

Using the calcium phosphate precipitate procedure, we have compared the relative number of stable, transformed colonies appearing on plates using extrachromosomal and integrating vectors. Using calcium phosphate precipitates, the transformation frequency with integrating vectors in these experiments was 20-50 per plate of 10^7 initial cells. Colonies can be normally seen at 5-7 days after selection has begun and stably

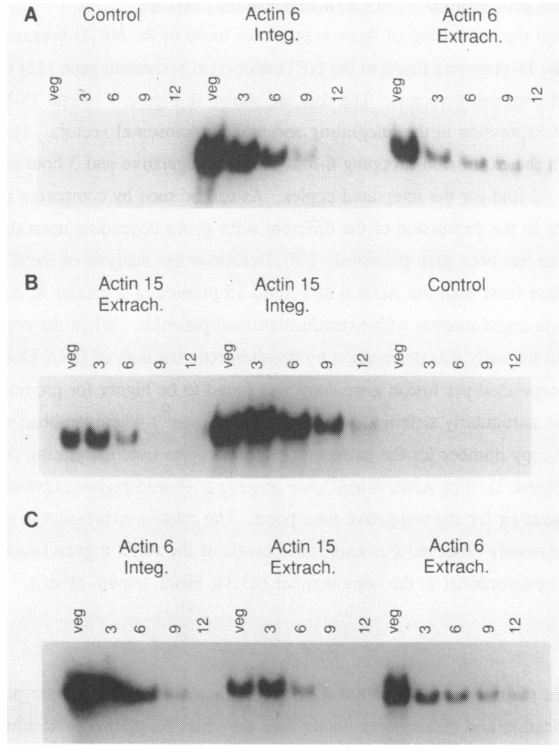

Figure 3. Comparative expression of the Actin 15 and Actin 6 promoter gene fusions on integrated and extrachromosomal DNA molecules. Vectors pA15TX (12,13) and A15E-A6 were used for the Actin 15 gene fusions and Vector I, B10SX, and VIE11 were used for Actin 6 gene fusion. To measure Actin 15-NPTI (neo^R) gene expression, the probe was made from the 1 kb *ClaI-SpeI* fragment spanning the NPTI resistance marker into the Actin 15 terminator that is common to the two molecules. To measure Actin 6-neo^R gene expression, the probe consisted of the internal 800 bp *PstI* from the NPTII gene. 7 µg of total cellular RNA was loaded in each lane. Control is untransformed cells. In panel C, the same filter was probed with both NPTI and NPTII probes. RNA and DNA levels were quantitated by densitometer. The levels of DNA were measured relative to CI and were as follows: B10SX=30.2, VIE11=1.0, A15E-A6=0.71, Vector I=11.1, and pA15TX=26.7. Relative expression normalized to vector copy number quantitated for these transformants as described in Materials and Methods.

transformed populations growing in shaking cultures can be obtained in 2-3 weeks (17; unpub. obser.). All the extrachromosomal vectors yielded 2-5-fold higher frequency of transformation and confluent plates of stably transformed cells can be obtained within 7-10 days (data not shown).

We have established protocols to electroporate DNA into *Dictyostelium* cells for transient assays (29). When electroporation was used to introduce integrating vector DNA into cells, few (<10) G418 resistant transformants are observed. However, all the extrachromosomal vectors tested gave a high number of transformants (several fold over calcium phosphate mediated transformation) and colonies were visible in 4-5 days (data not shown; ref. 29).

Expression of actin-neo gene fusions from extrachromosomal vectors

We have examined the expression of Actin 6 promoter fused to the NPTII neomycin resistance gene (10,11) and of the Actin 15 promoter fused to the NPTI neomycin resistance gene (12) on both extrachromosomally replicating and integrating vectors. The data are shown in Figure 3. Actin 15-NPTI (neo^R) gene fusion has a similar pattern of expression in the integrating and extrachromosomal vectors. The Actin 6-neo^R gene shows a difference with the expression dropping 6-fold between vegetative and 3 hour cells for the extrachromosomal copies and only ~2-fold for the integrated copies. As can be seen by comparing panels A and B to panel C, there are variabilities in the expression of the different actin genes depending upon the individual experiment and culture conditions as has been seen previously (36). Densitometric analysis of the RNA blots in Figure 3 show that total expression from both the Actin 6 and Actin 15 promoters is greater in the transformants carrying integrated vectors than in transformants with extrachromosomal plasmids. When the copy number of each actin-neo gene fusion in the cells was determined by densitometric tracings of DNA blot (data not shown), the relative level of RNA expressed per fusion gene copy was found to be higher for promoters on extrachromosomal plasmids. This is particularly striking for Actin 15-NPTI (neo^R) where we observe a 10-15-fold higher level of expression per copy number for the extrachromosomal copies over integrating copies (quantitation not shown; see legend to Figure 3). For Actin 6-neo^R, we observe a ~4-fold higher expression per copy of extrachromosomal over integrating for the vegetative time point. The relative expression is about the same for the 3 hour cells. We have previously observed that expression levels of the Actin 6 gene fusion and other gene fusions in integrating vectors is proportional to the copy number (23,37; Firtel, unpub. obser.).

DISCUSSION

Our data represent the only analysis of the regions of an endogenous eukaryotic plasmid necessary for extrachromosomal replication and maintenance, other than the yeast 2μ plasmid. Results indicate that the region required for extrachromosomal replication and stable maintenance is present on a ~2.2 kb *Hin*dIII fragment. It should be noted that we cannot rule out the possibility that the Ddp1-derived plasmids do not replicate extrachromosomally, but in fact jump into and out of the host genome as it replicates. We think this is unlikely because if this were so, we would always detect some integrated vector, and we do not. In transformants where we detect integrated and extrachromosomal copies, the copy number of those vectors in the cells was low (see Table I). These vectors may not replicate as efficiently as Ddp1-20. We believe these copies are stably integrated into these genomes. While we cannot exclude the possibility that vector sequences shuttle between integrated and extrachromosomal forms in these cells, we believe mutations in the origin or impairment of the origin function due to surrounding DNA sequences may allow for stable integration of a fraction of the molecules.

From the analysis of our constructs, other regions of the vector do not appear to be essential for replication or maintenance functions although vectors having additional segments of Ddp1 have a higher copy number (see Results). While the 2.2 kb *Hin*dIII fragment alone confers the ability to replicate extrachromosomally, an anomaly is observed: namely, the size of molecules containing it. These plasmids appeared to run as dimers (Figures 2 and 3). Interestingly, there is an observed shortening in the amount of time required to obtain stable transformants when the 3.5 kb region between the *Bgl*II and *Bam*HI (Cassette G; see Ddp1-20 map, Figure 1) sites was retained in the vector introduced by either the standard calcium phosphate protocol or by electroporation (unpublished observation) and the copy number was higher. The effect was not observed, however, when the *Bam*HI/*Bgl*II fragment alone was carried on integrating vectors (unpub. obser.). In addition, we found that

transformation with extrachromosomal vectors gives a stable transformed cell population in a substantially shorter time period than transformation with vectors that integrate. This difference may be due to extrachromosomally regulating vectors establishing themselves in the nucleus more rapidly than integrating vectors (10).

Although we have not rigorously tested the stability of these vectors, they appear to be stably maintained. Spores were made of each individual transformant, stored for several weeks at 4°C adhered to silica gel, germinated in axenic medium lacking G418, and grown for several generations. The cells were then placed into medium containing G418. Cells continued to grow logarithmically with little or no lag indicating that the vector is stable in a majority of the cells. Analysis of DNA isolated from these cells showed that the vector DNA was extrachromosomal. These results were also observed for all vectors that replicate extrachromosomally and there was no relationship between copy number and stability (data not shown).

Transcription units expressed during growth and development have been mapped to restriction fragments of Ddp1 (26). It is interesting that the region of Ddp1-20 that we define for extrachromosomal replication and stable maintenance does not contain any complete transcription units expressed during growth. Part of the G1 transcriptional unit expressed during growth is found within this region (26) as is the 3' end of the developmental transcript d5 (26; Powell and Firtel, unpub. obser.). It is not known if the region of G1 contains the promoter or 3' end of the gene. The developmental transcript d3 is encoded by Cassette C which also contains the promoter is d5 (26; Noegel, pers. commun.; Powell and Firtel, unpub. obser.). This suggests that the plasmid itself does not encode any gene products that are essential for the replication of the plasmid and that these plasmid transcripts presumably encode proteins with other functions. Moreover, since the host strain for these experiments contains no sequences that cross-hybridize with Ddp1, we assume that functions normally required for plasmid replication are provided by the host genome and that these host genes do not share extensive sequence homology with Ddp1 encoded genes. Ddp1, however, appears to impart no observable phenotype or selective advantage, at least in the laboratory, to cells harboring it. In the case of the yeast 2μ circle, there are several plasmid encoded gene products necessary for the proper plasmid maintenance, replication, and segregation, and they also regulate plasmid copy number (38-43). We observe differences in copy number and relative stability of plasmids with regard to deletions or apparent formation of dimers depending upon the Ddp1 sequences and their organization on the vectors. Whether or not Ddp1 encoded proteins play a role in these molecular phenotypes is not known.

Vectors without the *KpnI-BglII* region from Ddp1 form integrating stable transformants with low relative efficiency when the DNA is introduced by electroporation and high relative efficiency when the DNA is introduced in a calcium phosphate precipitate. Other results indicate that introduction of DNA into the cells is not the limiting factor in obtaining high frequencies of stable transformants by electroporation of integrating vectors. Transient assays of cells in which an Actin 15-firefly luciferase gene fusion was introduced indicate that during the first 10-48 hours after electroporation, comparable amounts of luciferase activity were obtained whether the fusion was carried on integrating or extrachromosomal vectors (29).

Interestingly, the relative level of expression per vector copy is higher in populations of transformants of extrachromosomally replicating vectors versus vectors that are integrated. We do not know the reason for this but it may be associated with the sites of integration for non-extrachromosomally replicating vectors, a function of the fact that the copies are in a tandem array, or some other mechanism. We do not feel this observation is a consequence of the fact that we are assaying the gene encoding the selectable marker since extrachromosomal vectors with apparently intrinsically different copy numbers express different levels of Actin 6-neo^R mRNA but all confer the same relative resistance to G418. In addition, we observe substantially different copy numbers of integrating vectors from population to populations; however, they all show similar G418 resistance. Moreover,

when Actin 6-neo^R fusion genes carrying mutations in the Actin 6 promoter that reduce the level of expression of the NPTII gene ~10-fold are examined, copy number of such plasmids in transformants is very similar to that of vectors carrying the wild-type Actin 6-neo^R gene fusions (14). In other studies, we have examined the expression of *Dd-ras* promoter-gene fusion in response to cAMP on integrating (linked to B10SX) and extrachromosomal (on Ddp1-20) vectors. We observe the same induction pattern for the *ras* promoter fusion between integrating and extrachromosomal vectors and the relative expression per gene copy is several-fold higher for the extrachromosomal vectors (Firtel and Silan, unpub. obser.).

Our analysis now permits a more detailed examination of the functions of the 2.2 kb *Hin*dIII fragment as a stable origin and also allows the construction of smaller vectors for use in expression of gene products or studies of promoter regions in *Dictyostelium*. The ability to obtain stable transformants at a higher frequency, more rapidly, and by the use of electroporation as well as calcium phosphate mediated transformation expands the approaches for understanding gene regulation in this organism. One clear advantage of extrachromosomal vectors is that genes expressed on these will not be influenced by the location in which the gene integrates within the host chromosome. In addition, the higher frequency of transformation makes practical experiments such as complementation of known mutants in *Dictyostelium* using a genomic DNA library. It may be possible to do shotgun mutagenesis in which antisense RNAs are produced downstream from specific promoters. Shuttle vectors carrying these DNAs would be easy to recover by transforming *E. coli* with crude, whole cell *Dictyostelium* DNA (1).

Further analysis of extrachromosomal vectors may define regions required for extrachromosomal replication in more detail. A number of other endogenous plasmids have been identified in a variety of *Dictyostelium discoideum* isolates and in other *Dictyostelium* species (10,25-27,44). Similar types of analyses with these plasmids in comparison with the results obtained here should further elucidate functions required for extrachromosomal replication in these organisms.

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