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Extrachromosomal Replication of Shuttle Vectors in *Dictyostelium discoideum*

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We cloned a 12.3-kilobase (kb) endogenous plasmid, Ddp1, found in several wild-type and laboratory strains of *Dictyostelium discoideum* into pBR322. The cloned plasmids have been used to cotransform *D. discoideum* cells with B10S, a transformation vector carrying a gene fusion conferring resistance to G418. Whereas B10S DNA alone appears to integrate in a tandem array, the cloned Ddp1 plasmids replicate extrachromosomally and are stably maintained in the absence of selection with an average copy number of 50 to 100 copies per cell. The Ddp1-derived plasmids can be directly recovered by transforming *Escherichia coli* with bulk nuclear DNA from these cells. Preliminary deletion analysis indicates that not all regions of Ddp1 are necessary for stable replication in *D. discoideum*. Several recombinant vectors which replicate extrachromosomally in *D. discoideum* were also isolated. One contains the Act6-neo^r gene fusion from B10S recombined into one of the cloned derivatives of Ddp1 and can be used to directly transform *D. discoideum* amoebae, selecting for G418 resistance. Another recombinant is only 5.6 kb and resulted from a deletion of a 16.6-kb cloned Ddp1 hybrid plasmid. An analysis of the vector DNAs present in clones derived from single *D. discoideum* transformants is also described.

Dictyostelium discoideum grows as single-celled amoebae. Upon starvation, the cells initiate a multicellular developmental program in which approximately 10^5 cells form an aggregate which then differentiates into a fruiting body containing predominantly two cell types: spores and stalk cells (7). We and others have been interested in understanding the mechanisms by which cell-type differentiation is regulated in this relatively simple multicellular system. To this end, genes which are expressed at specific developmental stages were cloned and used to examine the biochemical and physiological factors which regulate their expression. These genes include those expressed during vegetative growth and repressed during early development, genes expressed under various conditions during preaggregation, and the cell-type-specific genes that are preferentially expressed in either prestalk or prespore cells (1, 9; for a review, see reference 4). Several factors have been identified which are required for the expression of sets of coordinately regulated genes, including cAMP (3, 5, 8–10; S. Mann, R. A. Firtel, and J. Brandis, manuscript in preparation).

We have begun by examining the *cis*-acting DNA sequences that are involved in the proper developmental regulation of the expression of these genes and have established that there is a DNA-mediated transformation system in which cloned genes can be transformed into stably maintained and expressed *D. discoideum* (12, 13). Several genes which have been introduced into *D. discoideum* cells by this system have been shown to be transcribed from the proper cap site and regulated in a manner similar to that of their endogenous copies (13; C. Reymond, W. Nellen, and R. A. Firtel; Proc. Natl. Acad. Sci. USA, in press; W. Nellen and R. A. Firtel, Gene, in press). For several types of analyses, it would be advantageous to have a shuttle vector which would replicate extrachromosomally in *D. discoideum* cells and which could be easily recovered by transforming *Escherichia coli* with total cell DNA from *D. discoideum* tranformants or which could be purified from *D. discoideum* cells with their associated chromosomal proteins.

Several endogenous nuclear plasmids have been identified from at least three *D. discoideum* wild-type isolates and the laboratory strain HU32. All are found in multiple copies, and their sizes vary from approximately 7 to 25 kilobases (kb) (11; K. Williams personal communication; D. Welker, personal communication; R. A. Firtel and C. Reymond, unpublished data). The three plasmids that have been analyzed to date do not appear to cross-hybridize under standard conditons, suggesting that they do not share any major nucleotide sequence homology (R. A. Firtel, unpublished data). In this study we report the cloning of one of these plasmids, Ddp1, into pBR322 and show that the hybrid plasmid can be cotransformed into *D. discoideum* amoebae with vector B10S, a *D. discoideum* transformation vector carrying a fusion gene conferring resistance to the aminoglycoside G418 (13; Nellen and Firtel, in press). We find that transformants contain 50 to 100 copies of the hybrid *D. discoideum* plasmid per cell, that these plasmid DNA molecules replicate extrachromosomally, and that they can be recovered by transforming whole cell DNA directly into *E. coli*. In addition, we describe the isolation of several recombinant plasmids from the transformed *D. discoideum* cells.

MATERIALS AND METHODS

DNA-mediated transformation. DNA-mediated transformation was done as described previously (13) but with vector B10S, a derivative of vector B10 (12, 13) that contains the 3′ end of the *D. discoideum* actin 8 gene inserted 3′ to the neo^r

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gene (Nellen and Firtel, in press) (see Fig. 1). For cotransformations, equal masses of B10S and the cotransformed DNAs were used.

To grow transformed cells in G418, cells from the transformed population were inoculated at 2×10^5 to 5×10^5 cells per ml into HL5 medium containing 10 μg of G418 per ml. Once the cells started to grow (7 to 10 days), they were then transferred into medium containing 20 μg of G418 per ml. Cells capable of growing in G418 contain 50 to 150 copies of vector DNA per cell (Nellen and Firtel, in press). Results suggest that vector DNA (B10 or B10S) is integrated into the genome in a tandem array.

Clones derived from single cells were isolated by plating 10 to 100 cells on SM agar in the presence of *Klebsiella aerogenes* (8, 9). After colonies appeared on the plates, they were picked into axenic medium containing 250 μg of piperacillin and streptomycin sulfate per ml, with or without G418.

Isolation and cloning of Ddp1 plasmid DNA. Circular Ddp1 plasmid DNA was isolated from strain HU32 as described by Metz et al. (11). The DNA was digested with a series of restriction enzymes, and the sizes of the resulting products were determined on agarose gels. *Sph*I was shown to cut once within the vector, while *Bgl*II cut twice, with the sites being 0.8 kb apart. *Sph*I- or *Bgl*II-digested Ddp1 was cloned into the *Sph*I or *Bam*HI sites of pBR322, respectively, by standard protocols.

Analysis of DNA from transformants. DNA was isolated from transformants as described previously (13). The DNA was digested with restriction enzymes according to the direction of the manufacturer, except that RNase A and spermidine were added at concentrations of 1 μg/ml and 2.5 mM, respectively, during the digestions for DNA isolated by the miniprep procedure. This was not necessary for complete digestion of DNA purified on CsCl gradients.

DNA was size fractionated on agarose gels, blotted onto a Genentran membrane, and probed with nick-translated DNA probes as described previously (13). Restriction fragments for nick translation were gel purified twice on 0.8% preparative agarose gels, electroeluted into a trough, and further purified on 5 to 20% ethidium bromide-containing sucrose gradients to remove contaminants from the gel which inhibit the nick-translation reaction.

In experiments in which DNA from *D. discoideum* transformants was used to transform *E. coli*, the DNA was purified on CsCl gradients as described previously (13). Since total chromosomal DNA is known to inhibit the transformation frequency of *E. coli*, we mixed control plasmid DNA with DNA from untransformed cells at a 1:100 ratio to be able to more accurately quantitate the frequency of *E. coli* transformation with the DNA from *D. discoideum* transformants. Plasmid DNA from transformed *E. coli* colonies was isolated as described previously (2).

RESULTS

Cloning Ddp1. HU32, a derivative of the common *D. discoideum* wild-type strain NC-4, contains a plasmid, designated Ddp1, present in approximately 50 to 100 copies per cell (11). Similar, and possibly identical, plasmids have been found in NC-4 and another wild-type strain, V12 (11; R. A. Firtel, unpublished data). Ddp1 was isolated from HU32 and digested with a series of restriction enzymes to identify sites present only once. These experiments (data not shown) indicate that there is a unique *Sph*I site, which was used to clone Ddp1 into the *Sph*I site in pBR322 in both orientations, yielding plasmids pBMW3 and pBMW4. A restriction map of pBMW3 is shown in Fig. 1. Ddp1 was also digested with *Bgl*II and cloned in both orientations into the *Bam*HI site of pBR322, yielding hybrid plasmids pBMW1 and pBMW2 which carry a 0.8-kb deletion of the region between the two *Bgl*II sites (Fig. 1). Our restriction map indicates that Ddp1 is ~12.3 kb in contrast to an earlier report of ~13.5 kb (11).

Transforming Ddp1 containing hybrid plasmids into *D. discoideum* cells. We have described previously the construction of a transformation vector carrying the *D. discoideum* actin 6 promoter, 5′ flanking regions, and the neomycin resistance gene from Tn*5* which allows the selection of stable *D. discoideum* transformants resistant to G418 (12, 13). Transformants have, on the average, three to seven copies of vector DNA per cell arranged in an integrated, tandem array.

To determine if the cloned *D. discoideum* plasmid could be introduced and stably maintained in strain Ax-3 (6), an axenic cell line derived from NC-4 that does not harbor Ddp1 (11; R. A. Firtel, unpublished data), we cotransformed *D. discoideum* cells with the four cloned derivatives of Ddp1 and plasmid B10S (see above and Fig. 1), selecting for cells resistant to G418. Transformation frequencies ranged from 500 to 2,000 colonies per 10^7 cells with B10S alone or with B10S cotransformed with pBMW1, -2, -3, or -4. Control plasmids (pBR322 and pXF3) gave fewer than five colonies which, on subsequent analysis, were found to be sensitive to G418 (data not shown).

Of the transformed cultures that were obtained, three were used for a detailed analysis of the vector DNA in transformants. Culture 1, cotransformed with B10S plus pBMW3 and pBMW4, was grown in the absence of G418. Culture 2, transformed with B10S, was grown for several weeks in the presence of G418. Culture 4H, cotransformed with B10S plus pBMW3, was grown in the presence of G418. The DNA in cells from this experiment was examined after growth for 3, 5, and 9 weeks (denoted as cultures 4H3, 4H5, and 4H9, respectively). In Table 1 the properties of these cultures and some of the conclusions (see below) reached on the state of the vector DNA in these cells are summarized.

DNA was purified from populations of these and other cultures, and the size was determined on agarose gels directly or after it was digested with restriction enzymes. The DNA was blotted and hybridized to a purified restriction fragment from pBMW3 containing only Ddp1 complementary sequences, a purified fragment carrying the neo^r gene, or pBR322 to determine which vector DNAs were present in the cells and whether the DNA was integrated or extrachromosomal (Fig. 2 and 3). As can be seen from the hybridization results, sequences complementary to Ddp1 were detected in all lanes containing DNA from cell lines cotransformed with hybrid plasmids containing Ddp1 (cultures 1 and 4H), but not in other cell lines, indicating that pBMW3 and pBMW4 cotransformed into these cells (see Fig. 4). Analysis of DNA from another series of cell populations cotransformed with B10S and either pBMW3 or pBMW4 showed similar results (see below). Cotransformation with either pBMW1 or pBMW2 gave results similar to those with pBMW3 and pBMW4 (data not shown).

Hybrid plasmids containing Ddp1 are extrachromosomal in transformed cells. Previously we have shown that when undigested total cell DNA from cells transformed with vector B10 is sized on agarose gels, B10 sequences migrate with the bulk chromosomal DNA, which is consistent with the integration of DNA (13). To examine the state of the Ddp1 complementary sequences, DNAs from several cell

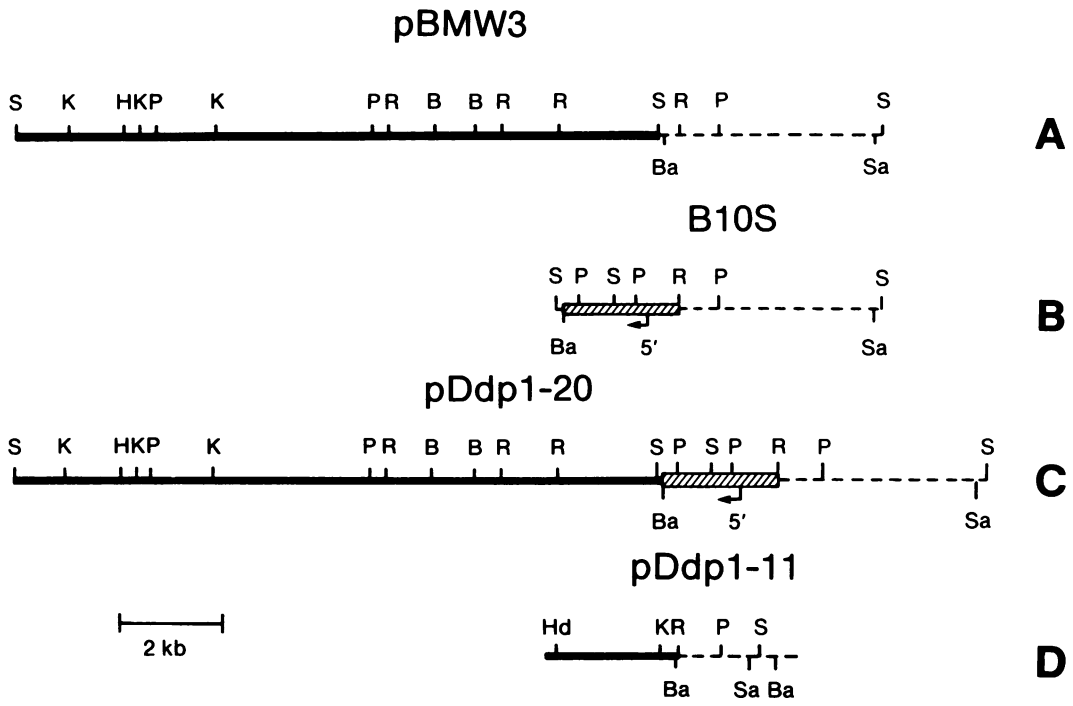


FIG. 1. Restriction map of plasmids. (A) pBMW3. Ddp1 was cloned into pBR322 at the unique *Sph*I site in both orientations, and a partial restriction map was derived. The major restriction fragments of cloned Ddp1 hybrid plasmids comigrate with those of the endogenous plasmid in strains HU32 and NC-4 (data not shown). The map of pBMW3 is shown. pBMW4 contains *Sph*I-linearized Ddp1 cloned in the opposite orientation into the *Sph*I site of pBR322. pBMW1 contains the large *Bgl*II fragment of Ddp1 cloned into the *Bam*HI site of pBR322 in the same orientation as in pBMW4 and lacks the small 0.8-kb *Bgl*II fragment. pBMW2 contains the large *Bgl*II fragment of Ddp1 cloned in the opposite orientation as in pBMW1. Ddp1 lacks sites for *Ava*I, *Bam*HI, *Bgl*I, *Mlv*I, *Pvu*I, *Sal*I, *Sma*I, *Sst*I, *Xho*I, and *Xma*III. A second *Hpa*I site may be present very close to the *Hpa*I site shown (T. E. Ward, unpublished data). (B) B10S. B10S carries the Act6-neo^r gene fusion from vector B10 and a restriction fragment from actin 8 carrying a transcription termination or polyadenylation signal or both (12, 13; Nellen and Firtel, in press). Its construction has been described previously (Nellen and Firtel, in press). (C) pDdp1-20. Restriction map of pDdp1-20 was constructed by analyzing restriction digests and DNA blots of the hybrid plasmid DNA isolated from *E. coli* and whole cell DNA from *D. discoideum* 4H5 which harbors the hybrid plasmid. pBR322, pBMW3, and the neo^r gene fragment were used as hybridization probes for the DNA blots used in constructing the map. (D) pDdp1-11. Restriction map of pDdp1-11 was constructed by analyzing restriction digests and DNA blots of the hybrid plasmid isolated from *E. coli*. pBMW3 and pBR322 were used as hybridization probes. To identify the regions of Ddp1 contained within pDdp1-11, we hybridized nick-translated pDdp1-11 to restriction digests of pBMW3. The map reveals an ~1.2-kb deletion of pBR322 between the *Sal*I site and the colE1 origin. Transformation of *E. coli* and recovery of plasmid DNA demonstrates the presence of a functional Amp^r gene and origin of replication. A novel *Bam*HI site appears to the right of the *Eco*RI site near the point of Ddp1 DNA insertion into pBR322. Hybridization of a Ddp1-11 probe to pBMW3 DNA blots only shows hybridization to the 2.2-kb *Eco*RI-*Sph*I fragment, yet the Ddp1 portion of Ddp1-11 contains a *Kpn*I site which presumably comes from the other side of Ddp1 (see map in part A), suggesting multiple recombination events. Abbreviations: K, *Kpn*I; S, *Sph*I; P, *Pst*I; H, *Hpa*I; R, *Eco*RI; B, *Bgl*II; Ba, *Bam*HI; Sa, *Sal*I; Heavy line, DNA sequences derived from Ddp1; light dashed line, DNA sequences derived from pBR322; box with slash, the Act 6-neo^r gene (carrying the actin 8 3' end) which confers G418 resistance to transformed cells (12, 13).

lines which had been cotransformed with B10S and pBMW3 and pBMW4 were analyzed.

When DNA from cells transformed with B10S (culture 2) was digested with *Bam*HI (which cuts once within B10S or pBMW3), blotted, and probed with nick-translated pBR322 or a purified neo^r gene probe, a single band with the mobility of linearized B10S vector DNA (6.1 kb) was seen, as expected from previous results (Fig. 3, lane 2-B) (13). The lane carrying undigested DNA from this cell population shows a broad band which comigrates with the bulk chro-

TABLE 1. Transformed cultures

Culture	DNA used in transformation	Grown in G418	Vector DNA in cells[a]	DNA copy no.	DNA state
1	B10S plus pBMW3 and pBMW4	No	pBMW3, pBMW4, smaller plasmids pDdp1-11	~50	Extrachromosomal
2	B10S	Yes	B10S	~50	Integrated
4H5[b]	B10S plus pBMW3	Yes	pBMW3, pDdp1-20[c]	~100	Extrachromosomal

[a] After 5 weeks of growth.
[b] Culture 4H was examined after 3, 5, or 9 weeks and is referred to as 4H3, 4H5, or 4H9, respectively.
[c] Ddp1-20 is a plasmid resulting from an in vivo recombination of pBMW3 and B10S (see text).

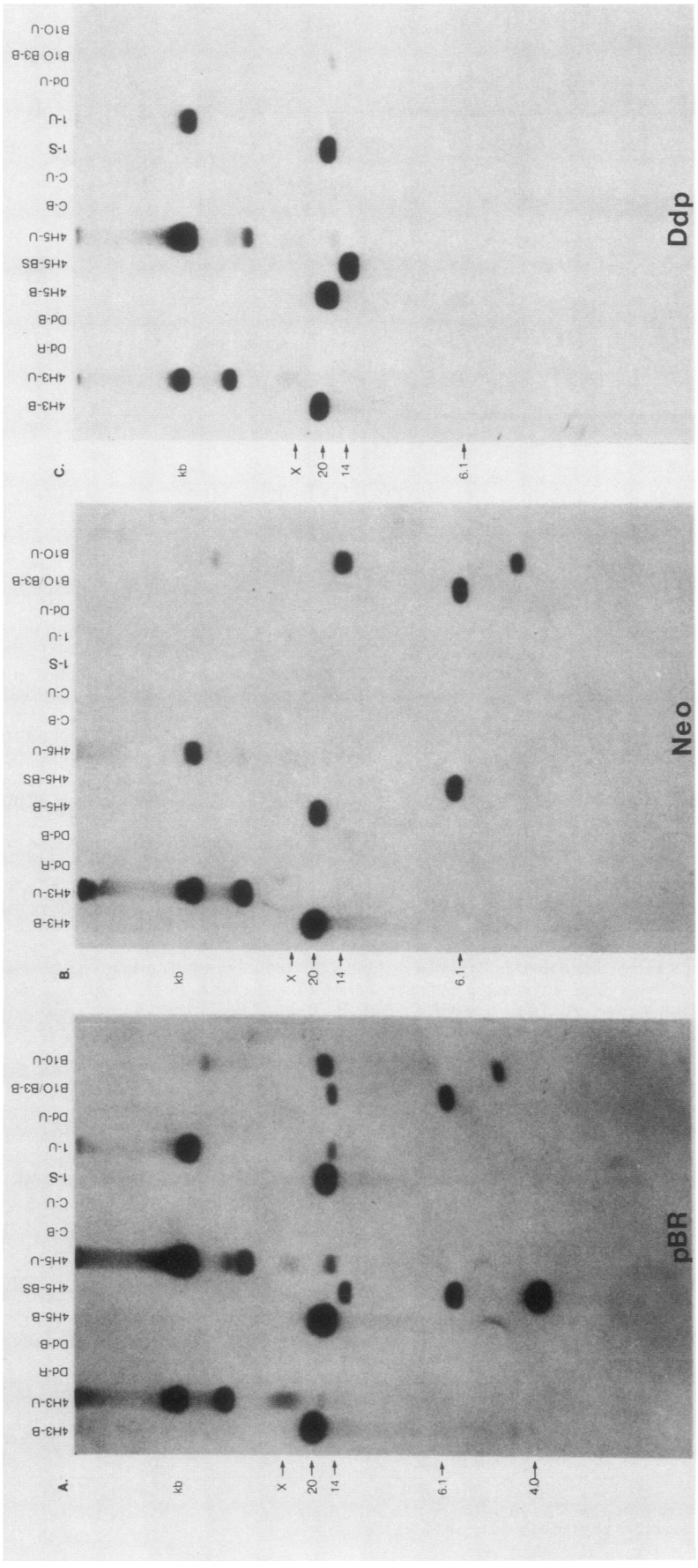


FIG. 2. Agarose gel of digested and undigested DNA from cells cotransformed with hybrid plasmids containing Ddp1 sequences. The gel (0.7%) was stained with ethidium bromide after electrophoresis to determine the position of prominent staining bands of known molecular weights. 4H3-B, DNA from culture 4H3 (Table 1) digested with *Bam*HI; 4H3-U, undigested DNA from 4H3; Dd-, DNA from *D. discoideum* Ax-3 cells digested with *Eco*RI (R), *Bam*HI (B), or undigested (U); 4H5, DNA from culture 4H5 digested with *Bam*HI (-B), *Bam*HI plus *Sal*I (-BS), or undigested (-U); C, DNA from mock-transformed cells not selected with G418 digested with *Bam*HI (-B) or undigested (-U); DNA from culture 1 (Table 1) digested with *Sal*I (-S) or undigested (-U); B10/B3-B, a mixture of B10S DNA and pBMW3 DNAs linearized with *Bam*HI at 20 genome equivalents (for the B10S) mixed with DNA from Ax-3 cells digested with *Eco*RI. B10-U, undigested B10S DNA, a mixture of closed and nicked circular forms, mixed with Ax-3 DNA digested with *Eco*RI prior to electrophoresis. The markers were added to digested Ax-3 DNA to prevent abnormalities in the mobilities of the DNAs which occurred occasionally when nanogram amounts of DNA were sized on these gels. (A) Gel probed with pBR322. (B) A duplicate gel to that shown in part A was blotted and probed with a purified restriction fragment containing the neomycin resistance gene from Tn*5*. (C) The labeled DNA on the blot shown in part A was removed with several washes of 2 mM EDTA (pH 8.4)–0.2% sodium dodecyl sulfate at 95°C, and the blot was reprobed with a purified nick-translated restriction fragment from pBMW3 (the large *Sph*I-*Eco*RI fragment; Fig. 1). The probe does not cross-hybridize to control *D. discoideum* DNA or to pBR322.

mosomal DNA (comparison of photographs of the stained gels and the DNA blots; data not shown). These results are consistent with our previous observations and suggest that the vector DNA is integrated in these cells in tandem arrays (13).

Cultures 4H3 and 4H5 were derived from cells cotransformed with B10S and pBMW3 and grown in the presence of G418 for either 3 (4H3) or 5 (4H5) weeks. When DNA from these cultures was digested with *Bam*HI and probed with pBR322, a single broad band was seen with a mobility slightly slower than that of linearized pBMW3 marker DNA (Fig. 2A, lane 4H3-B; Fig. 3A and C, lane 4H5-B). When undigested DNA from these cells was examined, a series of bands was observed, all of which had a mobility slower than that of bulk chromosomal DNA (Fig. 2A, lane 4H3-U; Fig. 3A and C, lane 4H5-U). These bands have approximately the same mobility as those bands seen when pBMW3 plasmid DNA isolated from *E. coli* is examined (Fig. 3, lane B3-U). This DNA runs in the position of nicked circular DNA and suggests that the DNA containing pBR322 complementary sequences from the cotransformed cells is extrachromosomal. The copy number was estimated to be approximately 50 copies per haploid genome by determining the hybridization intensity compared with those of standards (Fig. 2 and 3 and data not shown). A visible, ethidium bromide-stained band migrating in the position of the pBR322 hybridizing band was observed in lanes carrying undigested DNA from cells transformed with Ddp1 containing hybrid plasmids (data not shown). Such a band was not visible in lanes carrying DNA from cells transformed with B10S alone.

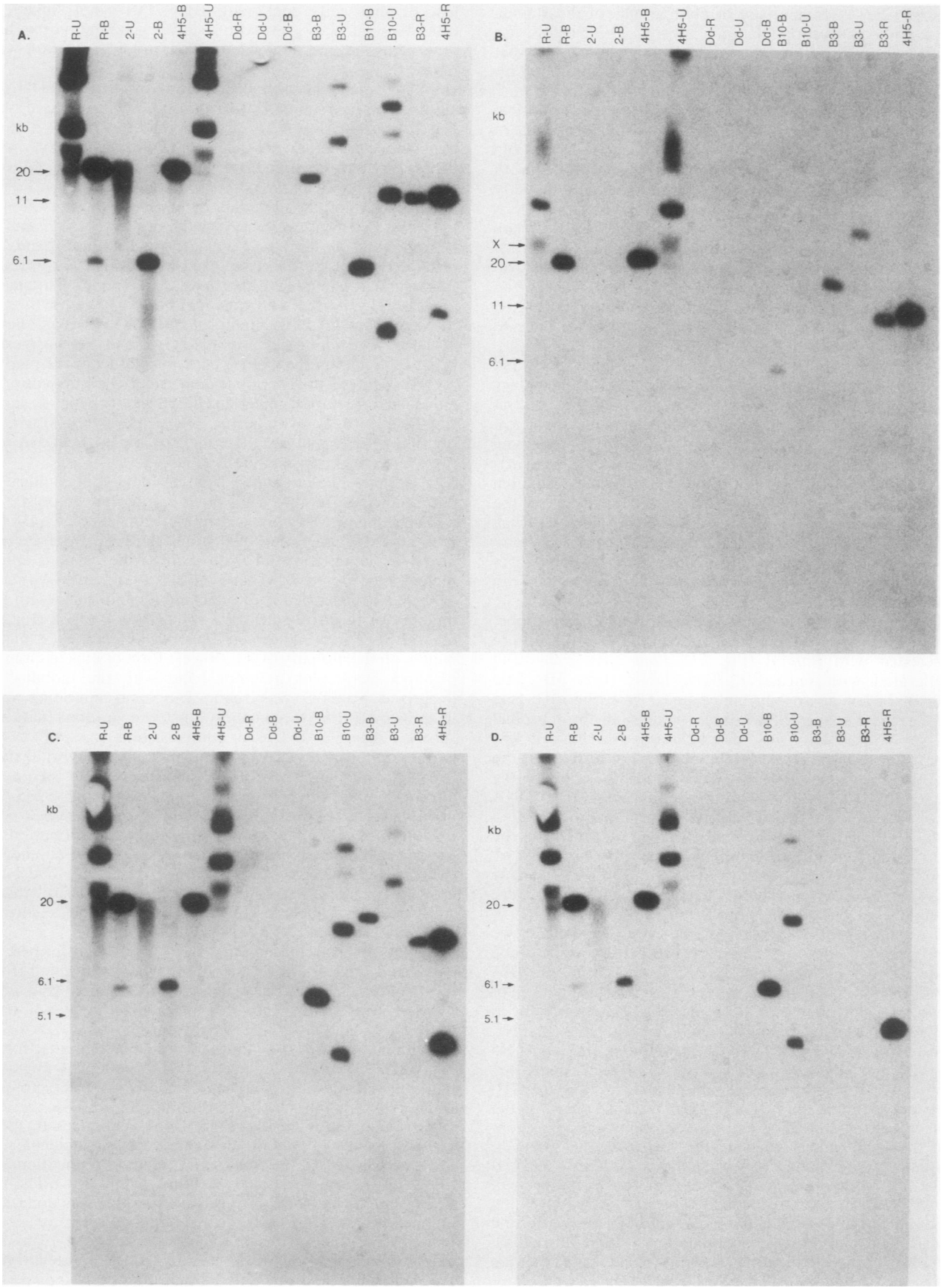
The blots were also hybridized with a neo^r gene probe. The probe hybridized to a 6.1-kb band in DNA from culture 2 digested with *Bam*HI (Fig. 3D, lane 2-B). This band comigrated with linearized B10S DNA (Fig. 3D, lane B10-B). In lanes carrying undigested DNA (Fig. 3D, lane 2-U), a smear comigrating with the undigested chromosomal DNA was seen. When DNA from cultures 4H3 and 4H5 was digested with *Bam*HI and probed with either pBR322 or the neo^r gene, no band was observed at the position of the linearized B10S DNA, indicating that unit-length B10S DNA is not present in these cultures. To confirm that the inability to detect the B10S-specific bands was not an artifact of the experiment, a mixture containing equal amounts of DNA from cultures 4H3 and 2H was analyzed. The B10S-specific bands as well as the high-molecular-weight bands were detected (Fig. 3A and C, lanes R-U and R-B).

To determine the location of the Ddp1 sequences, we hybridized the same or similar blots to a Ddp1-specific probe made from a purified restriction fragment from pBMW3 lacking pBR322 sequences. We observed the same pattern of hybridization with the Ddp1 probe as that observed with pBR322 or neo^r gene probes in lanes carrying *Bam*HI-digested or undigested DNA from cultures 4H3 and 4H5 (Fig. 2C and 3B). Since the neo^r gene, pBR322, and Ddp1 probes all hybridize to the same bands in the *Bam*HI-digested or undigested DNA lanes, at least some of the molecules carrying Ddp1 and pBR322 sequences also contain the neo^r gene sequence. This suggests that parts of B10S, including the neo^r gene, recombined into pBMW3 and that cells containing this DNA were selected during growth in G418 (see below). However, when DNA was isolated from the cotransformed cell population only 3 days after the transformed cells were harvested, a 6.1-kb B10S-specific band was observed (data not shown). This indicates that the B10S vector not linked to other exogenous DNA is later lost or that cells containing B10S DNA not linked to pBMW3 sequences grow with a slower generation time than other cells and are diluted out of the total population with time.

We investigated further the structure of the vector DNA in culture 4H5 by probing DNA from these cells digested with *Eco*RI or *Bam*HI-*Sal*I. Both digestions cut pBMW3 more than once. When *Eco*RI-digested DNA was probed with pBR322, two bands were observed, one which migrated with the same mobility as the corresponding band from pBMW3 vector DNA and an additional band of ~4.3 kb which also hybridized strongly to the neo^r gene probe (Fig. 3C and D, lane 4H5-R). A *Bam*HI-*Sal*I digest yielded three bands of ~12, ~6, and 4 kb that hybridized to pBR322. The ~12-kb fragment corresponded in size to the corresponding fragment of pBMW3, and the 4-kb fragment corresponded in size to the major pBR322 fragment of pBMW3 (Fig. 2, lanes 4H5-BS). The additional ~6-kb band which strongly hybridizes to the neo^r probe cannot be explained from the map of pBMW3. Interestingly, this fragment corresponds approximately in size to the linearized B10S vector (Fig. 2, lanes B10/B3-B). In *Eco*RI-digested DNA, the Ddp1 fragment hybridizes to a band with the same mobility in 4H5 DNA and pBMW3 control plasmid DNA (Fig. 3B).

DNA was also examined from a population (culture 1) cotransformed with B10S plus equimolar amounts of pBMW3 and pBMW4 and grown in the absence of G418 for 2 months. Hybridization of pBR322 or the Ddp1 fragment probe to DNA digested with *Sal*I (which has a single recognition site in B10S and pBMW3 and pBMW4) and analyzed as described above showed a major band with the mobility of linearized pBMW3 (or pBMW4) DNA (Fig. 2, lanes 1-S). When undigested DNA was examined, a major band with the mobility of the slowest form of nicked circular pBMW3 DNA plus a minor band with the mobility of linearized pBMW3 was seen. The band migrating with linearized pBMW3 (seen in only some preparations [data not shown]) may be due to a double-strand scission of the extrachromosomal DNA during isolation. No hybridization of DNA from these cells to the neo^r probe was seen even after extended autoradiography, indicating that B10S sequences were not present. When this transformed cell population was examined shortly after the transformation, it contained B10S sequences with an average copy number of two per cell (data not shown). Since these cells had been growing for more than 150 generations, it is possible that a subpopulation which lost B10S sequences had a shorter generation time and overgrew the entire culture.

Rescue of extrachromosomal DNA from transformed *D. discoideum* cells. We have shown that when undigested DNA from cultures 4H and 1 is analyzed, pBR322 or Ddp1 probes hybridized to molecules which migrated more slowly than the bulk of high-molecular-weight chromosomal DNA and with mobilities similar to those of the nicked circular forms of pBMW3. In contrast, vector DNA sequences migrated with the bulk chromosomal band when DNA was examined from cells transformed with B10S alone. These results suggest that the majority of the Ddp1 sequences in these cultures exist as extrachromosomal, circular molecules. If these molecules still carry a ColE1 origin of replication and the Amp^r gene, we should be able to directly transform *E.coli* with DNA from these cells. When this was done, the number of *E. coli* transformants obtained was consistent with the presence in the *D. discoideum* cells of 10 to 100 extrachromosomal molecules per haploid genome carrying the Amp^r gene and the ColE1 origin (data not shown, see


A.
R-U
R-B
2-U
2-B
4H5-B
4H5-U
Dd-R
Dd-U
Dd-B
B3-B
B3-U
B10-B
B10-U
B3-R
4H5-R
kb
20
11
6.1
B.
R-U
R-B
2-U
2-B
4H5-B
4H5-U
Dd-R
Dd-U
Dd-B
B10-B
B10-U
B3-B
B3-U
B3-R
4H5-R
kb
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6.1
C.
R-U
R-B
2-U
2-B
4H5-B
4H5-U
Dd-R
Dd-B
Dd-U
B10-B
B10-U
B3-B
B3-U
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4H5-R
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4H5-B
4H5-U
Dd-R
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Dd-U
B10-B
B10-U
B3-B
B3-U
B3-R
4H5-R
kb
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above). In contrast, DNA from culture 2 gave no *E. coli* transformants.

DNA from 36 Amp^r *E. coli* clones was digested with *Eco*RI, and the size was determined on agarose gels. A subset of these DNAs is shown in Fig. 4. DNA from *E. coli* transformed with DNA isolated from culture 1 showed a 3:1 ratio of colonies containing DNA with the same *Eco*RI restriction pattern as pBMW3 and pBMW4, respectively. In addition two clones carried a 5.6-kb plasmid which had only one *Eco*RI restriction site. This plasmid was designated pDdp1-11 (Fig. 4, lane c). All these DNAs hybridized to the pBMW3 probe but not to a neo^r probe, as was expected since the DNA was derived from culture 1 (Fig. 4; data not shown). A restriction map of pDdp1-11 is shown in Fig. 1D.

Isolation of a recombinant plasmid carrying the Act6-neo^r gene fusion and Ddp1. DNA from *E. coli* colonies transformed with DNA from culture 4H5 contained either DNA with the same *Eco*RI restriction pattern as pBMW3 or a new plasmid (pDdp1-20) containing an altered *Eco*RI restriction pattern. A series of minipreps of *E. coli* transformants gave a ratio of approximately 3:1 of pBMW3 to the new plasmid pDdp1-20. A subset is shown in Fig. 4, lanes d and e. Blots of plasmid DNA were hybridized with either the neo^r gene probe or to nick-translated pBMW3 (Fig. 4). As expected, pBMW3 hybridized well to all *Eco*RI fragments from the plasmid with the same restriction pattern as pBMW3. It also hybridized well to the three fragments of pDdp1-20 that had mobilities similar to those from pBMW3 and poorly to the 4.3-kb *Eco*RI fragment. The neo^r gene probe did not hybridize to the plasmid that had the same restriction pattern as pBMW3 but did hybridize strongly to the 4.3-kb fragments from pDdp1-20 and more weakly to the ~2.1-kb fragment (see legend to Fig. 4).

We mapped pDdp1-20 isolated from *E. coli* and *D. discoideum* cell line 4H5 using pBR322, pBMW3, and purified neo^r fragment as probes (data not shown). Results show that there was a homologous recombination event within the pBR322 sequences, resulting in the insertion of a single, complete Act6-neo^r gene fusion. A map of pDdp1-20 is shown in Fig. 1C. When this plasmid was digested with *Eco*RI-*Bam*HI, the fragment that showed the most intense hybridization to the neo^r gene probe had a similar mobility to the *Eco*RI-*Bam*HI fragment from B10S that contained the Act6-neo^r gene fusion (Fig. 4). These and other results indicate that all or parts of the neo^r gene integrate into pBMW3.

When DNA was analyzed from culture 4H grown for 9 weeks in G418, only extrachromosomal DNA with the restriction pattern of pDdp1-20 was identified (data not shown). When vector DNAs from this culture were rescued in *E. coli* and 36 colonies were examined, only plasmids with the same restriction pattern as pDdp1-20 were observed (data not shown). Analysis of DNA from clonal isolates showed that 11 of 12 appear to carry only pDdp1-20. A subset of these are shown in Fig. 5 (lanes e through j). One culture (Fig. 5, lane g) appeared to carry DNA that lacked several *Eco*RI fragments of pDdp1-20 or pBMW3. These results indicate that after extensive growth in G418, cells carrying pDdp1-20 are preferentially selected and that this plasmid is stable in *D. discoideum* cells under selection. These cells grow at the same rate as untransformed KAx-3.

pDdp1-20 was used to transform *D. discoideum* cells selecting for G418 resistance. The plasmid transforms and gives a frequency of transformation (as determined by the number of G418-resistant colonies obtained on filters [13]) >10-fold higher than that of B10S. In transformants, this DNA is extrachromosomal, as determined by DNA blot analysis and by the ability to transform *E. coli* (data not shown).

Analysis of vector DNA in clonal isolates of *D. discoideum* transformants. To examine whether transformed cells contain more than one form of the Ddp1-derived plasmid sequence, we analyzed DNA from clonal isolates of culture 1, which we have shown contains sequences with the same restriction maps as pBMW3 and pBMW4 and the recombinant 5.6-kb plasmid pDdp1-11. DNA was isolated from 25 single colonies, digested with *Eco*RI, and analyzed by DNA blot mappng with nick-translated pBMW3 as the hybridization probe. The analysis of the DNA from a selected set of clones is shown in Fig. 5 (lanes a through d). Sixteen of the clones contained DNA fragments with the same sizes as the *Eco*RI restriction fragments of pBMW3 (Fig. 5, lanes b and d). The other colonies contained fragments with mobilities of the *Eco*RI fragments of pBMW4 or pBMW4 plus several other high-molecular-weight bands (Fig. 5, lanes a and c). One of these bands was 5.6 kb and comigrated with the 5.6-kb plasmid pDdp1-11 isolated from *E. coli* transformants. *Bam*HI cleaved this plasmid into two fragments of ~2.8 kb which were also seen in digests of DNA from these colonies (data not shown; Fig. 1D). Additional *Eco*RI bands were also seen in DNA from clones carrying pDdp1-11 which did not comigrate with any *Eco*RI fragments of pBMW4. These have not been isolated in *E. coli* transformants, possibly because they are not linked to the Amp^r gene or contain an *E. coli* origin.

DISCUSSION

We cloned a 12.3-kb endogenous plasmid, Ddp1, from strain HU32, a derivative of the wild-type *D. discoideum* isolate NC4, into pBR322. These hybrid plasmids were cotransformed with vector B10S into an axenic strain lacking Ddp1. Analysis of transformants shows that the Ddp1 DNA sequences migrate in agarose gels as open circles, while in DNA isolated from cells transformed with B10S alone, vector sequencs comigrate with the bulk of chromosomal DNA. We also showed that bulk DNA from transformants containing the cloned Ddp1 can be used to directly transform

FIG. 3. DNA blot of size-fractionated DNAs from transformants. (A and B) Fractions of DNA samples were sized on 0.7% agarose gels, blotted, and probed with pBR322 (panel A) or the large *Sph*I-*Eco*RI restriction fragment from pBMW3 (panel B) (see legend to Fig. 2 for descriptions of lanes). (C and D) DNA samples were sized and blotted as described for those in panels A and B and probed with pBR322 (panel C). After autoradiography, the probe was stripped as described in the legend to Fig. 2, and the blot was then probed with a fragment containing the neo^r gene fragment (panel D). 2-U,B, DNAs from culture 2 (cells transformed with B10S and grown in the presence of G418); -U, undigested; -B digested with *Bam*HI; 4H5-, DNA from culture 4H5; B3-, pBMW3 marker DNA digested with *Bam*HI (-B), *Eco*RI (-R), or undigested (-U). Lanes R-U and R-B are reconstructions containing a 1:1 mixture of DNA from culture 2 and culture 4H5 either undigested (-U) or digested with *Bam*HI (-B). The other labels are the same as those described in the legend to Fig. 2; however, different DNA preparations were used for these gels.

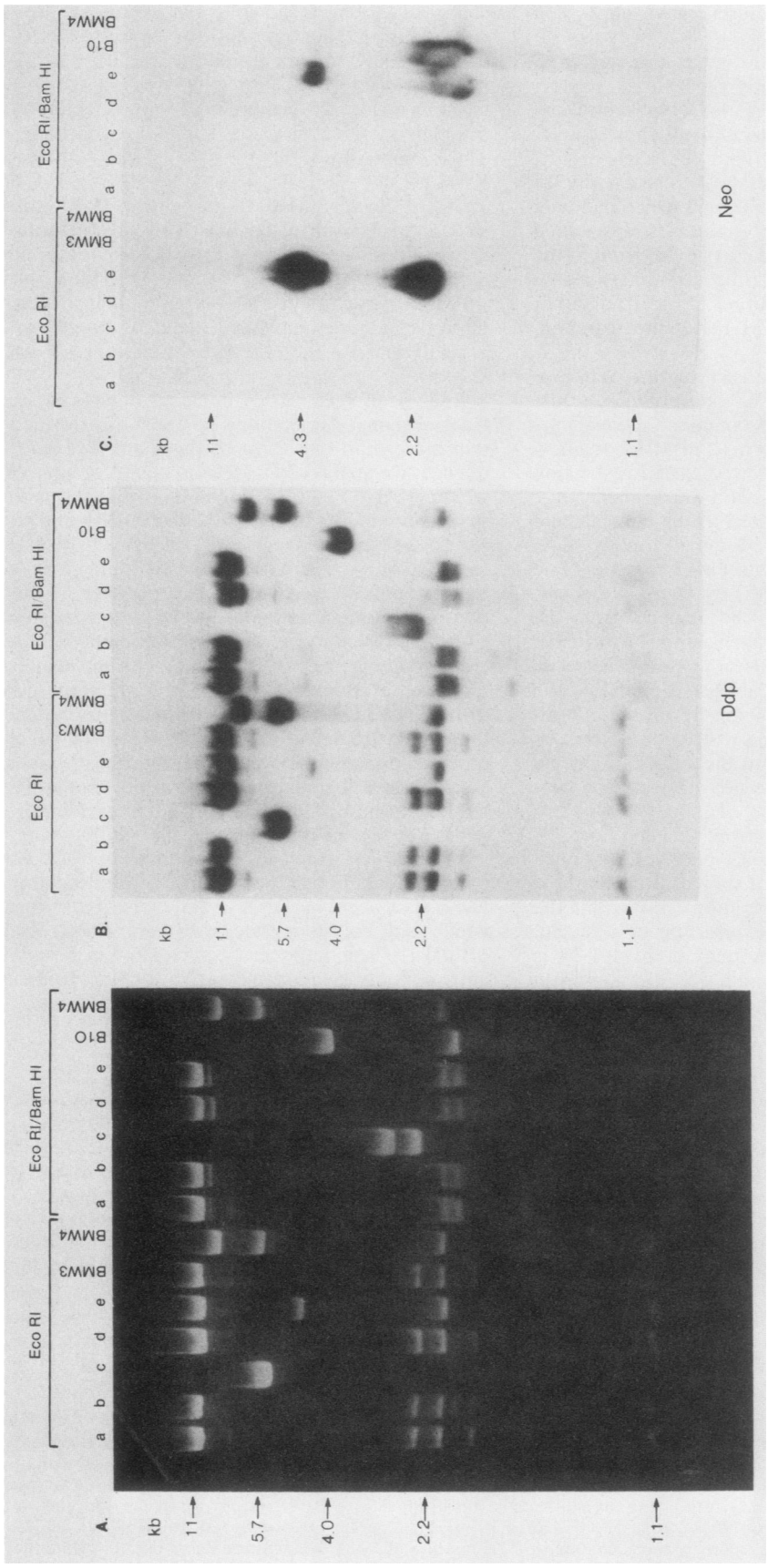

FIG. 4. Analysis of DNA from *E. coli* transformed with DNA from *D. discoideum* transformants. DNAs from selected *E. coli* clones transformed with DNA from culture 1 (lanes a through c) and culture 4H5 (lanes d and e) were digested with either *Eco*RI or *Eco*RI-*Bam*HI, and sizes were determined on a 0.8 % agarose gel. pBMW3, pBMW4, and B10S DNAs were used as markers. Lane c, plasmid Ddp1-11, the 5.7-kb plasmid obtained by transforming DNA from culture 1 into *E. coli*; lane e, pDdp1-20, the ~19-kb plasmid contains the Act6-neo^r gene fusion, obtained by transforming *E. coli* with DNA isolated from culture 4H5. (A) Ethidium bromide-stained gel. (B) Gel was blotted and probed with pBMW3. (C) The blot in panel B was stripped and reprobed with the neo^r gene fragment. The hybridization to the ~2.1-kb *Eco*RI fragment in lane e is due to the presence of a contaminating plasmid carrying a deletion in some pDdp1-20 preparations. This band has the identical mobility in our gel systems to the 2.1-kb *Eco*RI fragment in pDdp1-20 and contains neo^r gene sequences. A relativley low level of hybridization is seen to a 5.1-kb band in lane e of the *Eco*RI-*Bam*HI digest. This is probably due to a partial digest (probably of *Bam*HI) since this band has the same mobility as the band hybridizing to the neo^r gene probe in the *Eco*RI digest of this DNA and since no band with this mobility is seen in the stained gel.

E. coli, a result which is not obtained with DNA from B10S transformants. Together these results suggest that the majority of the Ddp1 sequences replicate extrachromosomally in transformants. No molecules running with the mobility of closed, circular, supercoiled pBMW3 DNA were observed. However, we expect that the extrachromosomal DNA is present as supercoiled molecules in *D. discoideum* cells, since the endogenous plasmid is known to be supercoiled in strain HU32 (11); we presume that the 16.6-kb pBMW3 molecule is nicked during isolation.

When DNA from culture 1 (cotransformed with pBMW3 and pBMW4, grown in the absence of G418) was used to transform *E. coli*, we obtained colonies containing plasmid DNA which had the same restriction pattern as pBMW3, pBMW4, or pBMW4 plus a new 5.6-kb plasmid, pDdp1-11. This smaller plasmid is seen in clones containing pBMW4, but not in clones containing pBMW3, and thus we expect that it arose by a deletion or a rearrangement of pBMW4 or both. Since this plasmid was recovered by directly using bulk chromosomal DNA to transform *E. coli*, we expect that this rearranged plasmid can replicate autonomously in *D. discoideum* cells.

Ddp1 was cloned into pBR322 in two different restriction sites in both orientations. We utilized the unique *Sph*I sites in both DNAs and also ligated *Bgl*II-digested Ddp1 into the *Bam*HI site of pBR322. The cloning into the *Bam*HI site results in the deletion of approximately 0.8 kb between the two *Bgl*II sites in Ddp1 (Fig. 1A). All four constructs are capable of replication when cotransformed into *D. discoideum*, indicating that pBR322 sequences do not interrupt essential functions for either replication or stability of the plasmid at either cloning site and that the 0.8-kb *Bgl*II fragment does not contain essential sequences. In complementary experiments, the 0.8-kb *Bgl*II in pBMW3 was deleted, and the resultant hybrid plasmid was cotransformed into *D. discoideum*. This hybrid plasmid was found to be stably maintained and extrachromosomal. We also deleted the two small, adjacent *Kpn*I restriction fragments from pBMW3 and pBMW4. These deleted hybrid plasmids can also be cotransformed into *D. discoideum* and replicate extrachromosomally (data not shown). The copy number was similar in all cultures examined. Cell lines were maintained for more than 9 months in the absence of any selection and continued to maintain the plasmids in more than 95% of the cells (R. A. Firtel and C. Silan, unpublished data).

When *D. discoideum* cells were cotransformed with B10S and pBMW3 and then selected for growth in axenic medium with G418 for several weeks (cultures 4H3 and 4H5), we obtained a mixture of plasmids when this DNA was used to transform *E. coli*. One plasmid had the same restriction map as pBMW3, while another had an altered restriction pattern and contained two *Eco*RI fragments which contained sequences complementary to the neo^r gene (pDdp1-20). When this DNA was digested with *Sph*I, *Pst*I, or *Eco*RI-*Bam*HI, fragments were obtained which comigrated with fragments from B10S carrying the Act6-neo^r gene fusion (data not shown). This and other mapping data have shown that pDdp1-20 carries the entire Act6-neo^r gene. This is supported by the fact that this plasmid can be used to directly transform *D. discoideum* cells and confers resistance to G418 and that the 1.2-kb mRNA produced from the Act6-neo^r gene fusion is expressed in these cells at high levels (R. A. Firtel, unpublished data). We propose that pBMW3 and B10S recombine within the *D. discoideum* cells at a low to moderate frequency, probably by homologous recombination between the pBR322 sequences in both plasmids.

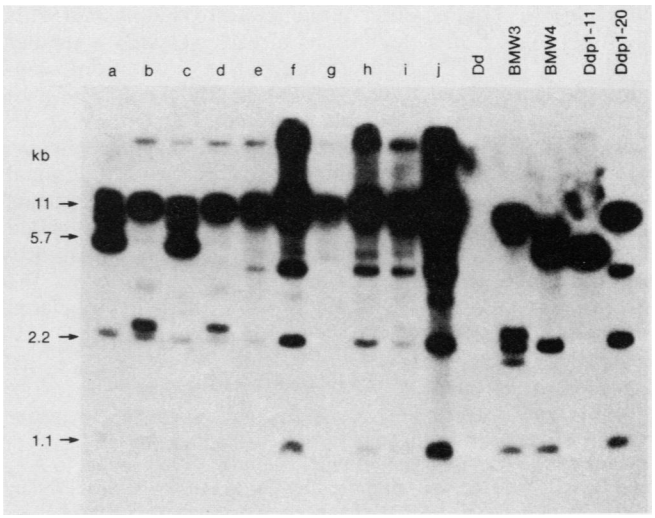

FIG. 5. Analysis of DNAs from clonal isolates. DNA from a selected set of clones derived from single cells was digested with *Eco*RI, sized on a 0.8% agarose gel, blotted, and probed with nick-translated pBMW3. Markers are pBMW3, pBMW4, Ddp1-11, and Ddp1-20 (50 copy equivalents per genome) digested with *Eco*RI. Lanes a through d, DNA from clones derived from culture 1; lanes e through j, DNA from culture 4H9 (grown for 9 weeks in the presence of G418).

When the selective pressure of growth in axenic medium containing G418 was applied, cells carrying the recombined vector grew at a faster rate and took over the population. This is supported by our observation that most single colonies of *D. discoideum* transformants after 9 weeks of growth contained only pDdp1-20, while pBMW3 was found in cultures grown for 3 or 5 weeks, and B10S sequences were detected only shortly after the transformation.

Recently, we used B10S-derived plasmids carrying *D. discoideum* developmentally regulated gene fusions in cotransformation experiments with pBMW3. When these were grown under selective conditions, cell lines were obtained which contained recombined extrachromosomal DNA carrying Ddp1 and pBR322, along with the Act6-neo^r fusion and the *D. discoideum* developmentally regulated gene fusion isolated by transforming *E. coli* with total *D. discoideum* DNA (R. A. Firtel and C. Silan, unpublished data). Like Ddp1-20, these apparently arise from recombination and suggest that similar cotransformation experiments can be used to obtain cell lines carrying extrachromosomally replicating fusion plasmids carrying a desired developmentally regulated gene fusion.

We showed that hybrid plasmids containing Ddp1 can be cotransformed into *D. discoideum* cells with B10S selecting for resistance to G418, that the DNA replicates extrachromosomally, and that the DNA can be used to directly transform *E. coli* to recover the transformation vectors. Recently, we have also shown that other DNAs (non-*Dictyostelium* plasmid DNAs cloned in *E. coli*) can also be cotransformed into *D. discoideum* (Nellen and Firtel, submitted). Cotransformation of Ddp1-derived vectors with B10S will be useful as a shuttle vector system to transfer sequences directly between *D. discoideum* and *E. coli*. Modification of the pBMW vectors to carry the Act6-neo^r gene fusion directly linked to Ddp1 sequences and the deletion of sequences unnecessary for plasmid replication and maintenance in *D. discoideum* should give us more

flexibility for cloning other sequences into the plasmid. It is possible that *D. discoideum* has already selected a smaller vector (pDdp1-11) capable of replicating extrachromosomally. We also showed that pDdp1-20, the vector isolated from culture 4H5, is capable of directly transforming *D. discoideum* cells to G418 resistance and that it replicates extrachromosomally, suggesting that it carries a functional Act6-neo^r gene fusion. We expect that these plasmids will allow us to examine the control of developmentally regulated genes on a multicopy vector which does not apparently integrate into the chromosomes. It may also allow the eventual isolation of regulated genes with their associated regulatory and other chromosomal proteins.

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