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An insertional mutagenesis approach to *Dictyostelium* cell death

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Abstract

Programmed cell death (PCD) in *Dictyostelium* shows a pattern of ordered degeneration similar to that observed in higher eukaryotes but somewhat different from the most studied form of PCD, i.e. apoptosis. To contribute to a genetic definition of this process, *Dictyostelium* HMX44A cells have been subjected to insertional mutagenesis, followed by selection based on several rounds of differentiation/regrowth to recover only cells resistant to death. We describe here the approach used, a partial characterization of the first mutant thus obtained called C5 showing some dissociation of cell death signs, and, in this case where plasmid rescue was not possible, as a first step towards identification of the gene at play recovery of genomic flanking sequences via genomic recircularization and PCR. This work demonstrates the feasibility of an insertional mutagenesis approach to obtain death-resistant mutants in *Dictyostelium*.

Keywords: cell death; *Dictyostelium*; insertional mutagenesis; evolution

Abbreviations: PCD, programmed cell death; DIF-1, differentiation inducing factor-1; cAMP, cyclic adenosine-3′-5′ monophosphate; FDA, fluorescein diacetate; PI, propidium iodide; Ca^{2+}, calcium

Introduction

Programmed cell death (PCD), which is essential for proper development and functioning of organisms (Glücksmann, 1951), seems to occur throughout evolution from primitive eukaryotic organisms to multicellular animals and plants (reviewed in Greenberg, 1996; Jacobson *et al*, 1997). To study more precisely the extent of conservation of PCD throughout evolution, we resorted to *Dictyostelium discoideum*. This protist is one of the currently surviving, most anciently diverged eukaryotic organisms (Field *et al*, 1988), stemming from the root of the terminal crown, perhaps after divergence of the kingdom Plantae and before individualization of the kingdoms Animalia and Fungi (see for instance Baldauf and Doolittle, 1997). *Dictyostelium* multiplies as a unicellular organism under favorable conditions but becomes multicellular by aggregation upon starvation (Raper, 1984). Aggregation is followed with cell differentiation and morphogenesis, leading within 24 h to the formation of a 1–2 mm high fungus-like structure called sorocarp. The sorocarp comprises two main populations of cells: viable spores in a bolus, and vacuolated cells tightly compacted in a stalk. Stalk cells have been shown to be non-viable, as they do not regrow in culture medium (Whittingham and Raper, 1960). Stalk cells thus may be considered an example of developmental PCD.

Dictyostelium discoideum is an attractive model to study PCD. Differentiation is simple, ultimately leading to only two main types of cells (viable spore and dead stalk cells). The main inducers of differentiation are known, including in particular: cAMP (cyclic Adenosine-3′-5 Monophosphate), the dichlorinated hexanone DIF-1 (Differentiation Inducing Factor-1) that readily passes the plasma membrane (Town *et al*, 1976; Town and Stanford, 1979; Sobolewski *et al*, 1983; Morris *et al*, 1987) and ammonia via pH variations (see Gross, 1994 for a review). Recent studies showed that the inducing effect of DIF-1 on stalk cell differentiation is mediated by a slow sustained increase in calcium (Ca^{2+}) levels (Schaap *et al*, 1996), and involves transcriptional activation of a STAT (Signal Transducer and Activator of Transcription) protein (Kawata *et al*, 1997). There might be about 300 genes involved in differentiation (Loomis, 1980) out of a total of 7000 to 17 000 genes for the whole 40 Mb genome (Sharp and Devine, 1989; Loomis and Smith, 1995). Powerful genetic techniques have been developed to allow identification and analysis of such genes (Kuspa and Loomis, 1992; Chang *et al*, 1995; Spann *et al*, 1996; see Kuspa *et al*, 1995 for a review), which is made easier by the haploidy of *Dictyostelium*.

In order to characterize PCD in *Dictyostelium*, and to avoid the morphogenesis step that leads to the formation of a stalk impairing direct microscopic observation of the corresponding cells, we took advantage of a protocol of differentiation in monolayer (Kay, 1987) of a *Dictyostelium* mutant strain called HM44 (Kopachik *et al*, 1983) derived for axenic growth as HMX44 (JG Williams, University College, London). HMX44 produces very little DIF-1 but is sensitive to added DIF (Kopachik *et al*, 1983). Upon starvation and addition of DIF-1, it differentiates into stalk cells, without however morphogenizing into a sorocarp. We thus could observe and characterize the morphological signs of *Dictyostelium* (stalk) cell death (Cornillon *et al*, 1994). In *Dictyostelium* PCD 50% of the cells do not regrow after about 8 h of DIF-1 action, and there is cytoplasmic and some chromatin condensation, massive vacuolization at 12 h and late membrane permeabilization. This pattern of degeneration is similar to what has been observed in higher eukaryotes with regards for instance to vacuolization, cytoplasmic condensation (Clarke, 1990) and

Ca^{2+} level modifications (Schaap *et al*, 1996), but somewhat different from the most studied form of PCD, i.e. apoptosis (Kerr *et al*, 1972; Wyllie, 1980), notably by the absence of DNA fragmentation (Cornillon *et al*, 1994). Our further preliminary experiments indicate that HMX44 cell death cannot be blocked by a range of cysteine-protease and of macromolecular synthesis inhibitors. Does the apparent relative morphological conservation of PCD correspond to conservation of at least some of the genes underlying this process?

In order to identify genes involved in *Dictyostelium* PCD, we attempted to isolate cell death mutants generated by the insertional mutagenesis bsr-REMI approach. REMI (for Restriction Enzyme Mediated Integration) consists in the introduction into cells of a mix of a restricted linearized plasmid and a related restriction enzyme (Kuspa and Loomis, 1992). As a result, the plasmid integrates into the genome with increased frequency at any potential site corresponding to the added enzyme (Kuspa and Loomis, 1992). The bsr-REMI approach makes use of a plasmid bearing a gene that confers resistance to the antibiotic blasticidin, allowing easy selection of eukaryotic cells after plasmid integration (Adachi *et al*, 1994). Plasmid integration may occur in one of the genes coding for proteins required for *Dictyostelium* PCD, eventually resulting in mutants with a cell death-resistant phenotype. These mutants can then be isolated through their survival upon DIF induction of death. In practice, however, since 10–20% of parental untransformed cells would survive under our selection conditions, we had to resort to progressive enrichment of mutant death-resistant cells by successive differentiation and re-growth experiments to allow potential mutants to emerge from this normal background of non-dying cells. A mutant partially resistant to DIF-induced cell death called C5 has been isolated. As a main phenotypic characteristic, after incubation in starvation medium either in the absence of DIF or at any concentration of DIF-1 up to 200 nM, the C5 mutant showed significantly more re-growth than the parental HMX44A cells. In the presence of DIF-1, it showed somewhat less vacuolization than the parental cells, and some delay in the transcription of the prestalk *ecmA* and *ecmB* genes, which however clearly took place. Sequences flanking the inserted plasmid could be isolated by recircularization/PCR. These results show the feasibility of this insertional mutagenesis approach to isolate cell-death-affected variants and the corresponding crippled genes.

Results

Insertional mutagenesis, screening, and isolation of the C5 mutant

Forty-six independent REMI transformations were performed with HMX44A cells using the pUCBsrΔ*Bam*HI vector (Adachi *et al*, 1994) (Figure 1A). Transformation efficiencies were 2×10^{-5} to 1×10^{-6} (not shown). After 10–11 days of blasticidin selection, equal numbers of cells from transformed populations were pooled 2 by 2, subjected to the DIF-1 death-inducing signal, and tested for their ability to re-grow. As even untransfected cells yield a background of 10–20% of non-dying cells under our differentiation conditions, to allow possible death-resistant mutant cells to emerge from the background and thus be detected each pool of transfected cells was subjected to up to seven successive rounds of the basic differentiation/re-growth protocol (Cornillon *et al*, 1994). Untransfected HMX44A cells were subjected to the same treatment and served as a control.

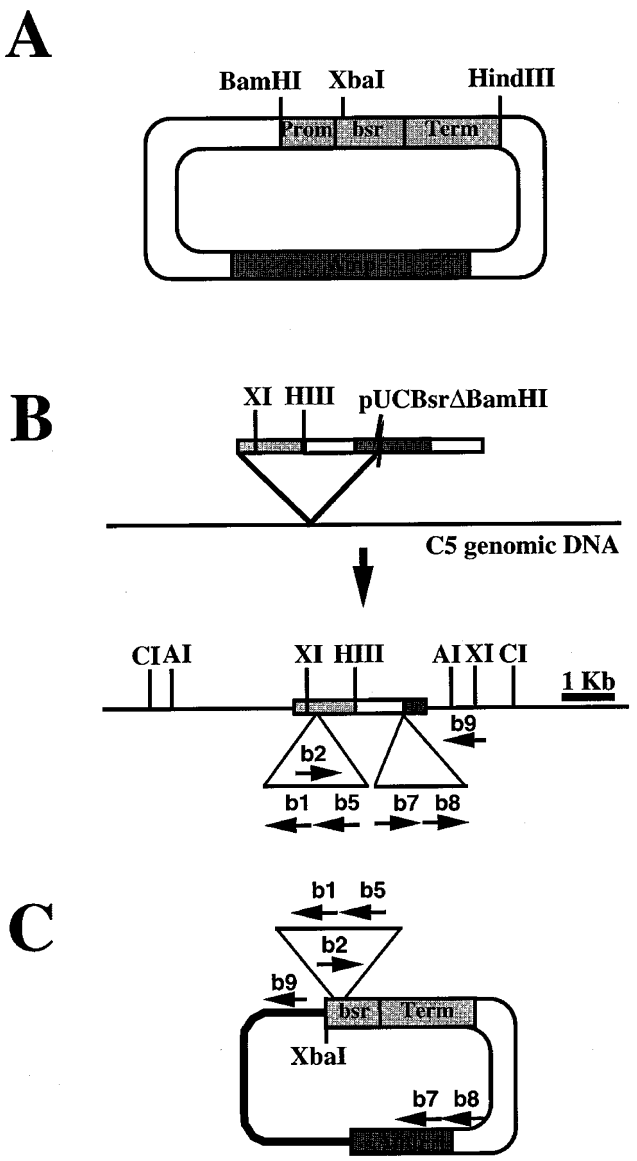

Figure 1 (**A**) The pUCBsrΔ*Bam*HI vector (not to scale), adapted from (Adachi *et al*, 1994). 'Prom'=*Dictyostelium* Actin 15 promoter; 'bsr'=blasticidin coding sequence; 'Term'=*Dictyostelium* Actin 8 terminator; 'Amp'=ampicillin resistance cassette. (**B**) Diagram of the incomplete integration of pUCBsrΔ*Bam*HI vector in C5 genomic DNA. *Cl*=*Cla*I; AI=*Acc*I; *H*III=*Hind*III. Positions of *Xba*I sites (XI) in the genome and in the vector are indicated. (**C**) Recircularized C5 genomic *Xba*I restriction fragment including part of the vector (not to scale). Genomic part of the ligated DNA is shown as a black line. Position of the primers used for the recovery of the right flank sequence by PCR is indicated by arrows

Figure 2A shows the result of one of these selections. Values are expressed as the ratio of [total number of transformed cells ('bR') after indicated rounds of differentiation/re-growth/total number of untransformed cells ('HMX44A') in the same conditions]. Two distinct pools of blasticidin-resistant cells, (bR15+16) and (bR17+18) were subjected to DIF-1 selection and counted after the re-growth step of each differentiation/re-growth round of selection. After each of the first few rounds, (bR15+16) and (bR17+18) showed background ratios of less than 10. At the end of the 4th round, cells within the (bR15+16) population started to emerge from the background resulting in a ratio of about 20. This was confirmed after the next round when the ratio for (bR15+16) reached about 130. Thus, only five rounds were necessary to detect a mutant population in (bR15+16). Some cells within this population were then cloned and subjected to a further step of differentiation/re-growth. As shown in Figure 2B, each of the clones tested had a death-resistant phenotype, showing a ratio about 4–6 times higher than that of the control, indicating that death-resistant mutant cells constituted the vast majority of the surviving transformants in (bR15+16). This was also observed in Southern blot analysis using DNA from the whole of the (bR15+16) population at the end of the five rounds of selection, and DNA from clone no 5 of Figure 2B. Genomic DNAs were ClaI digested, and the blots obtained were probed with pUCBsrΔ*Bam*HI. In this experiment, only one main band was observed for (bR15+16) genomic DNA (Figure 3A) showing that this population selected for resistance to death was homogenous as to the site of plasmid insertion within the *Dictyostelium* genome. This argues that this insertion was responsible for the selected death-resistant phenotype (see Discussion). Clone no 5 (C5) also bore this insertion, and was used for further experiments.

C5 bears a single insertion of a truncated vector and shows delayed transcription of *ecmA* and *ecmB* genes

Southern blot analysis on C5 with pUCBsrΔ*Bam*HI as a probe indicated through double digests (Figure 3A) that C5 bears a single insertion of the pUCBsrΔ*Bam*HI vector, and that only part of this vector was present in C5. The deduced restriction map of the inserted partial vector and the flanking C5 genomic sequences are shown in Figure 1B. The fact that only part of the vector (including in particular only a fraction of the ampicillin resistance cassette) was present in C5 prevented us from directly recovering genomic flanking sequences by plasmid rescue (Kuspa and Loomis, 1992). Northern blot analysis from a differentiation kinetic experiment indicated that in C5 the prestalk genes *ecmA* and *ecmB*, whose transcription is DIF-1 dependent (Williams *et al*, 1987; Jermyn *et al*, 1987) are transcribed upon addition of DIF-1, however less abundantly and with some delay when compared to HMX44A (Figure 3B).

C5 shows resistance to death in terms of regrowth

We checked first the regrowth properties of vegetative C5 *versus* untransformed HMX44A cells. As shown in Figure 4A, vegetative C5 and HMX44A cells have very similar growth kinetics. This indicates that it is not because of faster growth in rich medium that C5 showed more re-grown cells after DIF-1 induction of cell death. After this induction, resistance to death in terms of more re-growing cells could also be the result of protection due for instance to high cell concentrations. This is not the case, as shown in Figure 4B: C5 resistance to death is not dependent on cell concentration during differentiation

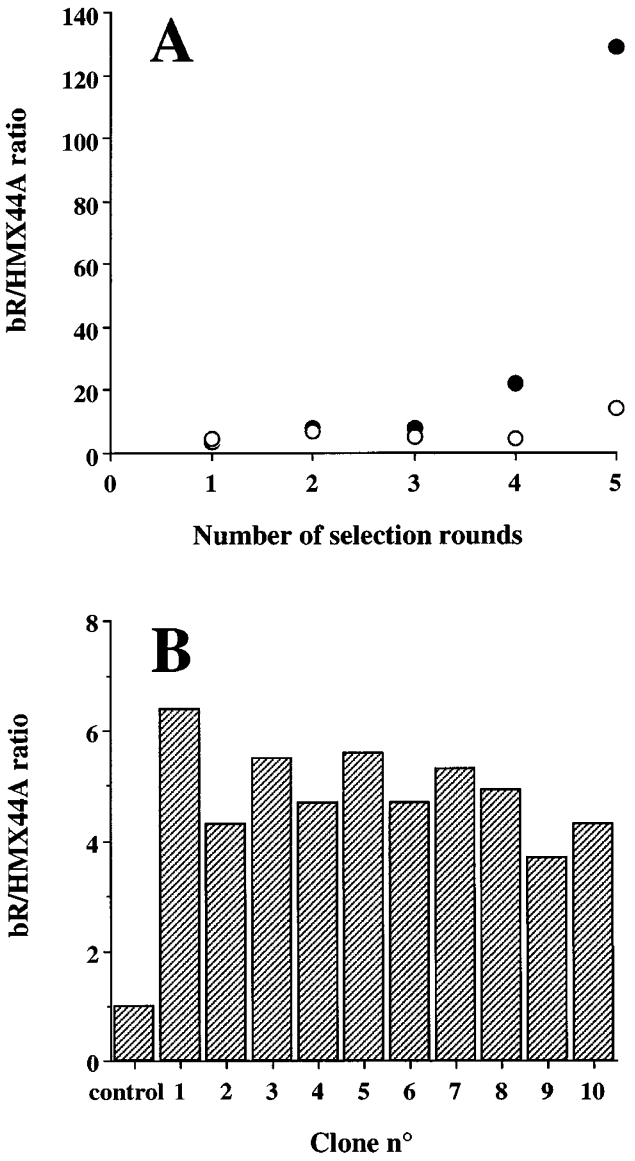

Figure 2 (**A**) Differentiation/re-growth selection on pooled transformed populations (bR15+16). (bR17+18) is another pool of transformed populations tested at the same time as (bR15+16). Values are expressed as the ratio of [the total number of transformed cells ('bR') after indicated rounds of differentiation/re-growth/the total number of untransformed cells ('HMX44A') in the same conditions]. ●: (bR15+16); ○: (bR17+18). (**B**) A single round of differentiation/re-growth for 10 clones from the (bR15+16) population with regards to untransformed cells. Values are expressed as for **A**. The discrepancy between Figure 2A and B concerning respective ratios for the (bR15+16) population at the end of the 5th round and for clones derived from it, comes from the fact that during the successive rounds of differentiation/re-growth, concentrations were not re-adjusted for the cells from the (bR15+16) population, leading to increasing accumulation of mutant cells resistant to cell death compared to the situation for the clones

within the range of cell densities tested. We also checked whether the properties of C5 could be related to delayed sensitivity to DIF or to particular DIF concentrations. Again in terms of re-growth, C5 resistance to death lasts for at least 72 h in the presence of DIF-1 (Figure 4C) and is independent of DIF concentrations as the DIF/SB cell count ratio after re-growth remained under all conditions 2–3-fold higher than that of HMX44A (Figure 4D). It should be noted that even in the absence of DIF, the resistance of C5 to SB treatment alone is about 3-fold higher than that of MMX44A (Figure 4D). Table 1 shows the results of each of the 19 such experiments we performed, comparing regrowth of HMX44A and C5. In parental HMX44A cells, incubation in SB alone led to marked decrease in the number of subsequently regrowing cells (results not shown). This was further decreased upon addition of DIF. C5 cells showed significantly more regrowth than HMX44A cells, after incubation in SB alone or with added DIF (Table 1).

C5 shows death in terms of vacuolization, however often delayed and less marked compared to parental HMX44A cells

Using a staining mix of fluorescein diacetate (FDA) and propidium iodide (PI) (Cornillon *et al*, 1994), *Dictyostelium* living cells (FDA+/PI−) can be distinguished from late dead cells (FDA-/PI+). As observed in Figure 5, after 12 h in the presence of DIF-1, HMX44A cells start to vacuolize. In contrast, C5 cells show no obvious vacuolization. After 24 h, while in SB alone HMX44A as well as C5 cells are unvacuolized and PI negative, in the presence of DIF most of the HMX44A cells are vacuolized and some show PI positivity while C5 cells show variable vacuolization sometimes marked as in the experiment shown here. Some C5 cells are positive for PI staining. Although within a C5 population subjected to DIF a number of cells show vacuolization, many cells would regrow in rich medium. We do not know yet which extent of vacuolization would prevent subsequent regrowth.

Recovery of genomic sequences flanking the inserted vector

As already mentioned and shown in Figure 1B, the pUCBsrΔ*Bam*HI vector has not been fully integrated (or has been rearranged) in C5, thus not allowing recovery of flanking sequences by classical plasmid rescue. We tried several other approaches to recover flanking sequences, including cloning of C5 genomic DNA in plasmid or phage vectors, and LMPCR (Balavoine, 1996), with no success. We then resorted to the following recircularization PCR approach (see also Pang and Knecht (1997) and references therein). C5 genomic DNA was XbaI digested, fractionated by gel electrophoresis to

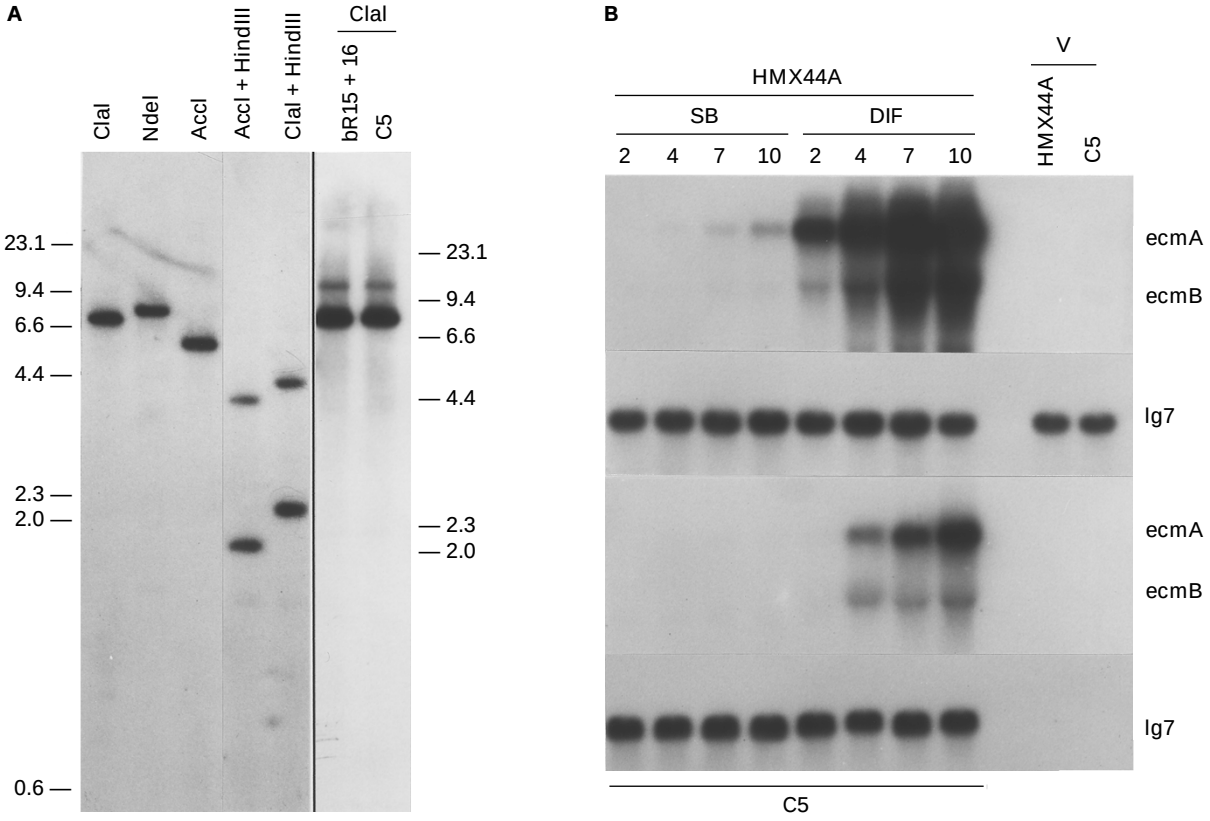

Figure 3 (**A**) Southern blots. DNA were digested with indicated enzymes and blots were probed with pUCBsrΔ*Bam*HI. Left part: C5 Southern blot. Right part: bR15+16 and C5 Southern blot. (**B**) Northern blot of a differentiation kinetic experiment on C5 (lower half) and HMX44A (upper half) cells. 'V'=RNA from vegetative cells. Cells were recovered for RNA extraction after the indicated number of hours in SB or SB+DIF. *EcmA* and *ecmB* cDNAs were used as probes. *Ig7* cDNA was used as a probe to check for quantities of RNA charged on gel. DIF/SB ratios (see Figure 4) of this experiment were 11% and 63% for HMX44A and C5, respectively

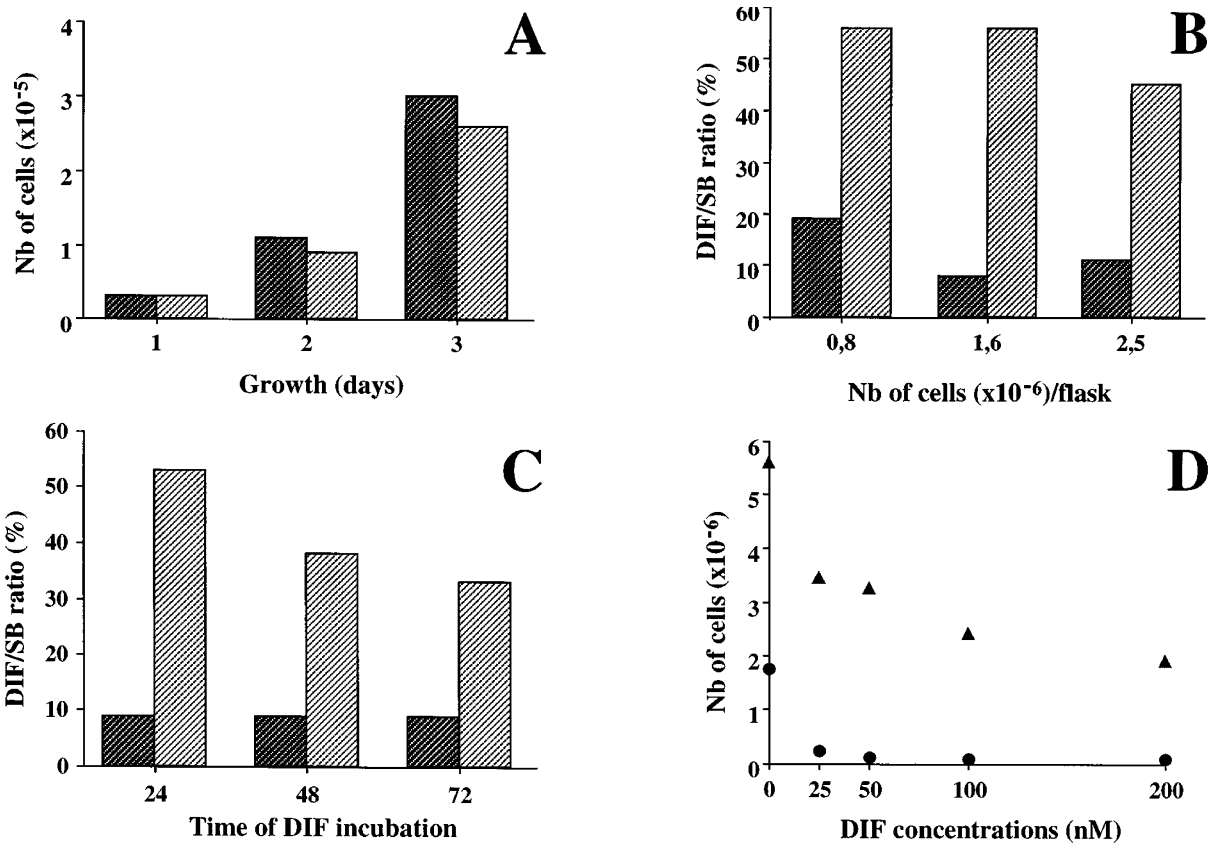

Figure 4 Regrowth properties of C5 mutant cells, using untransformed HMX44A cells as controls. (**A**) Growth kinetics of C5. Duplicate wells of 24 well plates (Falcon) each received 5×10^3 cells in 1.5 ml of HL5 medium (with blasticidin in the case of C5) at Day 0, and cells were counted after culture for 1, 2 or 3 days. This is one of two experiments with similar results. ▬: C5; ▨: HMX44A. (**B**) Effects of cell density on C5 resistance to death. Values are expressed as the ratio of [the total number of cells regrown after a differentiation/re-growth experiment in SB+DIF/the total number of cells regrown after the same differentiation/re-growth experiment in SB alone]. Cells were regrown in HL5 medium for 48 h before counting. This is one of two experiments with similar results. ▬: C5; ▨: HMX44A. (**C**) Effects on C5 of prolonged incubation in the presence of DIF. Cells were subjected to either 24 h, 48 h or 72 h of SB + DIF in this differentiation/re-growth experiment. Values are expressed as for Figure 3B. Cells were counted as for Figure 2B. This is one of two experiments with similar results. ▬: C5; ▨: HMX44A. (**D**) Effects on C5 of increasing concentrations of DIF. Cells were subjected to indicated DIF concentrations in this differentiation/re-growth experiment. Values are expressed as the total number of regrown cells after 48 h in HL5. This is one of two experiments with similar results. ▲: C5; ●: HMX44A

recover restriction fragments corresponding to the size of the *Xba*I band observed by Southern blot using the vector as a probe (note that this band includes part of the vector and, to its 'right', a genomic flanking sequence, Figure 1B), and purified (see Materials and Methods). An aliquot of this DNA was religated and used as a template with the oligonucleotides b8+b5 as primers in a PCR reaction (Figure 1C). The product thus obtained was used in turn as a template (see Materials and Methods) in a second PCR reaction with oligonucleotides b1+b7 as primers. The product of this second PCR reaction called 'right flank PCR product', was sequenced and allowed the design of the b9 primer. The right flank PCR product could thus be subsequently checked by direct PCR reaction with b7+b9 primers either on C5 or HMX44A undigested genomic DNAs (Figure 6A). Importantly, a single band of the same apparent size was observed when either C5 (but not HMX44A) genomic DNA or the right flank PCR product were used as a template with b7+b9 primers. Another PCR reaction was performed with b2+b9 primers on genomic DNAs and a single band of expected size with regard to the respective position of primers was also observed for C5 genomic DNA. These results clearly indicate that the PCR product we obtained by religation of the *Xba*I digested C5 genomic DNA reflects the genomic organization of C5 at the site of vector insertion.

In order to check for the presence of a transcribed gene in the right flank sequence, the PCR product was used as a probe on the Northern blot already probed with *ecmA* and *ecmB* (Figure 3B). A band of the same size and intensity was observed in all conditions tested, including also RNA from vegetative cells (Figure 6B). Sequencing results of the right flank DNA indicate the presence of several potential open reading frames, two of which would be able to code for proteins of about 80 amino acids. No clear homology was found with proteins in the available databases (not shown).

Table 1 Regrowth of parental or mutant cells after incubation in SB with or without DIF

	HMX44A		C5	
Exp. No	**SB**	**DIF**	**SB**	**DIF**
1	2.5	0.6	6.3	3.0
2	1.8	0.1	5.6	2.4
3	1.8	0.2	6.5	3.4
4	4.1	1.4	7.4	5.4
5	2.3	0.2	6.4	3.5
6	2.6	0.5	7.0	3.9
7	2.3	0.3	5.6	3.2
8	5.8	1.3	12.3	9.0
9	1.7	0.8	3.9	1.9
10	1.5	0.1	4.2	2.2
11	2.0	0.6	3.2	2.8
12	1.8	0.1	5.8	2.3
13	1.1	0.1	2.4	1.1
14	1.9	0.2	2.6	1.9
15	0.9	0.1	5.4	2.5
16	3.6	0.5	4.4	2.7
17	2.2	0.6	6.9	2.0
18	0.7	0.1	2.3	0.9
19	1.3	0.2	1.9	1.4
	100%	17%	261%	136%

Results of 19 independent differentiation/re-growth experiments with HMX44A and C5 cells. Cells were led to differentiate for 24 h (exp. 1–13) or 48 h (exp. 14–19) in SB with or without DIF, then 're-grown' for 48 h in rich medium (HL5). Figures are expressed as the number of cells: 10^6, determined at the end of this 48 h incubation. The bottom line represents averaged percentages for each column, taking for each experiment the number of HMX44A cells regrown after differentiation in SB alone as the 100% value

Discussion

To which extent is cell death conserved during evolution? To approach this question, we chose to study PCD in the protist *Dictyostelium discoideum*, one of the most ancient surviving organisms where cell death has been shown to occur in normal, developmental circumstances. *Dictyostelium* PCD presents morphological characteristics (Cornillon *et al*, 1994) similar to those observed in higher multicellular organisms though somewhat different from the most studied form of PCD, i.e. apoptosis. In order to isolate genes involved in *Dictyostelium* PCD, we used insertional mutagenesis coupled with selection based on enrichment of cells resistant to death. The latter took advantage of a method of differentiation in monolayers, allowing us to apply selection at the cellular level.

In this publication, we describe the methods used and show the isolation and preliminary characterization of a *Dictyostelium* cell death mutant. This mutant C5 is partially resistant to DIF-1-induced cell death. C5 may be altered in cell death signaling rather than mutated in a gene directly involved in the execution of cell death, since (1) *ecmA* and *ecmB* prestalk genes are transcribed with some delay and (2) differentiating C5 cells can show some vacuolization in the presence of DIF-1, including positivity in PI staining. Importantly, C5 cells are clearly able to re-grow much more than HMX44 cells. This resistance to starvation-induced conditions also occurs in the absence of added DIF-1, i.e. in SB alone. In starvation buffer the very small amounts of endogenous DIF-1 produced by HMX44A cells (Kopachik *et al*, 1983) were never sufficient to lead to detectable vacuolization. Still, we cannot entirely exclude the possibility that relative resistance in SB alone of C5, which is derived from HMX44A, may be related to its resistance to DIF-induced cell death. We tend to favor another scenario. Death in HMX44A cells might have two distinct components, perhaps each downstream of a distinct pathway. On the one hand, inhibition of growth would occur in SB alone. On the other hand gross morphological alterations including vacuolization would require further addition of DIF. While the alteration in C5 might reside upstream of both pathways, it would seem to affect more inhibition of growth than vacuolization. Whether better regrowth in this case means more cells regrowing or cells regrowing quicker is now under investigation.

Is the integration of the vector in the C5 genome responsible for the C5 phenotype? The cell population (bR15+16) from which C5 was cloned was homogenous with regard to the mutant cell death phenotype (Figure 2B), and with regard to the site of vector insertion, as assessed by Southern blot (Figure 3A). If another mutation, and not vector integration, were responsible for the phenotype, it then would have had to occur after insertional mutagenesis, during selection, which is unlikely although not formally excluded. This will be checked by homologous recombination and inactivation using the recovered and related genomic sequences.

Northern blot results obtained with the right flank PCR product as a probe show a band of the same size and intensity in parental HMX44A and in C5. Size similarity indicates that integration was not in this transcribed sequence. Intensity similarity indicates that integration did not functionally modify a regulatory region for this transcript. It is possible that integration took place in a regulatory region for a gene located further in the genome, leading to the cell death resistance phenotype observed. We are currently trying to isolate longer flanking sequences, on both sides of the integrated vector. The gene responsible for the observed phenotype may not be on the left side of the insertion, since the left side flanking sequence is quite poor in usual restriction enzyme sites (as indicated by further Southern blot analysis, not shown), suggesting a low G-C content and thus a low probability for the presence of coding sequences.

Several papers dealing with the characterization of *Dictyostelium* developmental mutants obtained by REMI have been published since the original publication by Kuspa and Loomis (see for instance Insall *et al*, 1994; Dynes *et al*, 1994; Segall *et al*, 1995; Harwood *et al*, 1995; Wang *et al*, 1996; Chang *et al*, 1996; Stege *et al*, 1997). REMI is thus a powerful genetic approach to isolate and characterize developmental mutants. It should be noted however that 10–20% of the mutants thus generated can be difficult to analyse because of deletions taking place near the point of vector integration (Kuspa *et al*, 1995), rearrangement or incomplete integration of the vector into the genome. In this case, ways of cloning flanking sequences other than plasmid rescue have to be set up and used, such as Partial Inverse PCR (Pang and Knecht, 1997) or the recircularization/PCR approach used here.

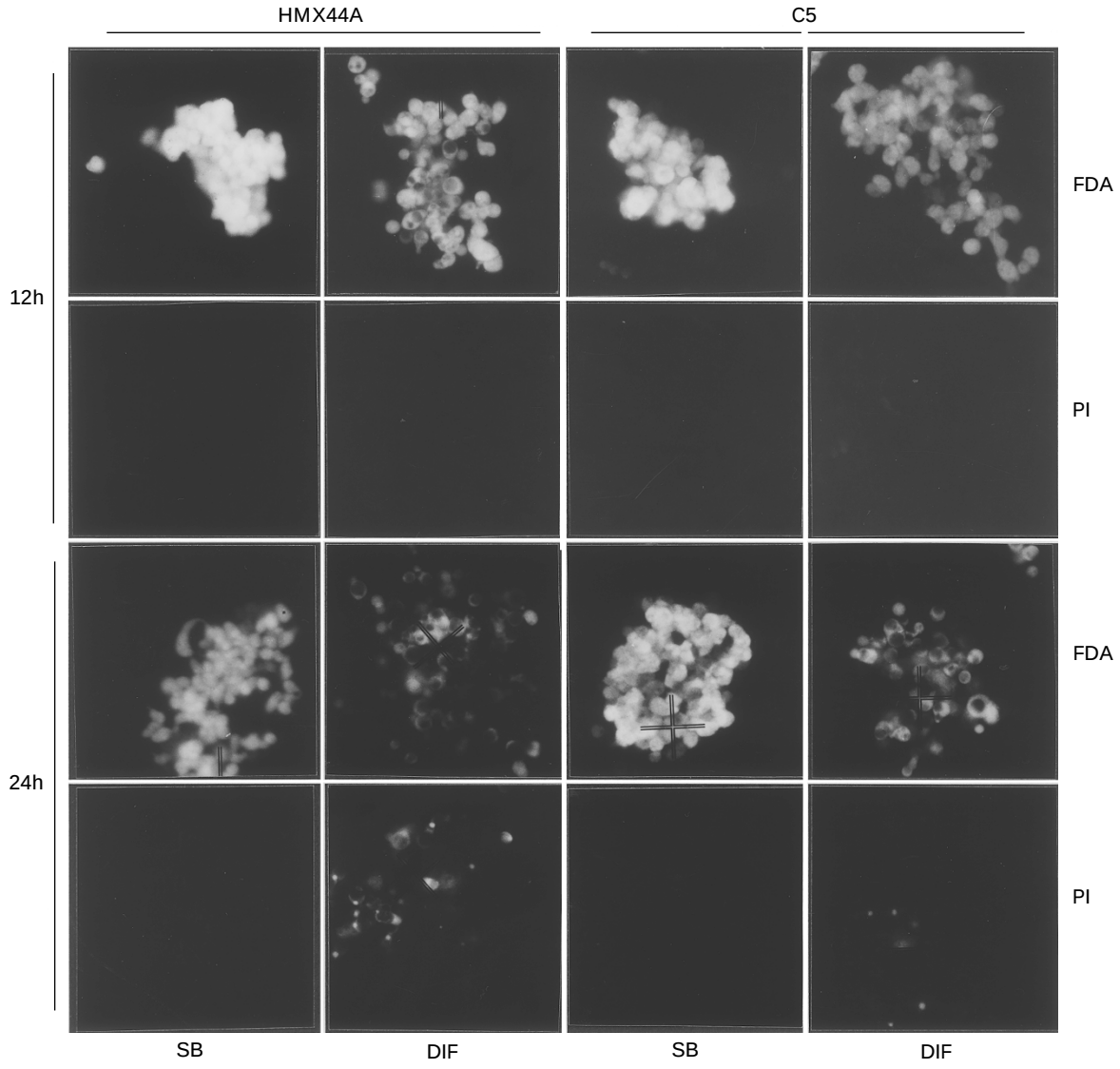

Figure 5 FDA (fluorescein diacetate)/PI (propidium iodide) staining on HMX44A cells and C5 cells differentiating for 12 or 24 h in the presence of DIF. Controls were cells in SB alone. DIF/SB ratios for 24 h in the presence of DIF, calculated by counting cells after 48 h of re-growth in HL5 medium, were 3% for HMX44A and 34% for C5, respectively

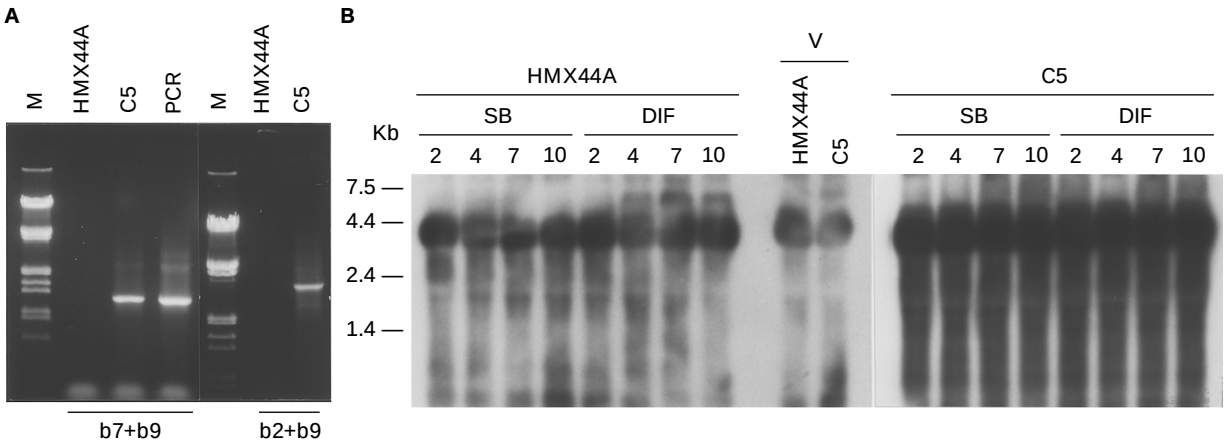

Figure 6 Gel electrophoresis of PCR products obtained with b7+b9 primers on C5 and HMX44A genomic DNAs, and on right flank PCR ('PCR') product. HMX44A genomic DNA was used as a negative control. 'M'=λ/*Hind*III+*Eco*RI. The approximate size of the PCR products is, for (b7+b9) 1.4 kb, for (b2+b9) 3 kb

This method was easy and rapid in our hands. It can lead within less than one week to the identification of flanking sequences when a mutant is potentially interesting but plasmid rescue is not possible. More generally, the present report on the C5 mutant, and the more recent isolation (not shown) of a second death mutant, shows the feasibility of this approach to isolate *Dictyostelium* cell death mutants generated by REMI.

Materials and Methods

Culture conditions

HMX44A, a subclone from HMX44 (a gift from JG Williams, University College, London) was used throughout. Cells were cultured in HL5 modified medium (Cornillon *et al*, 1994) in 50 ml flasks (Falcon, Becton Dickinson Labware, NJ) and subcultured twice a week in 10 ml of culture medium; 250 ml and 750 ml flasks (Falcon) were used when large numbers of cells were required. Cultures and experiments were at 22–23°C (Cornillon *et al*, 1994). Fresh cultures of HMX44A were started every sixth week from a large frozen stock.

Bsr-REMI transformants (see below) were cultured in the presence of 10 μg/ml blasticidin S hydrochloride (Calbiochem, Calbiochem-Novabiochem Corporation, La Jolla, CA). The blasticidin stock solution (4 mg/ml in milli-Q water) was filtered, and stored frozen at -20°C.

Transformation and drug selection procedures

HMX44A cells were transformed with the pUCBsrΔ*Bam*HI vector (Figure 1A, obtained from I Adachi, University of Tokyo) by the REMI procedure, according to Adachi *et al*, 1994) with slight modifications to the Adachi protocol. Briefly: logarithmically growing cells in HL5 were washed once in sterile ice cold electroporation buffer (10 mM NaPO4 pH 6.1, 50 mM sucrose), counted and resuspended at 50×10^6 cells/ml of the same buffer. The cell suspension (0.8 ml) in 0.4 cm gap electroporation cuvettes (Bio-Rad, Hercules, CA) with 10 μg of *Bam*HI linearized vector and 10 units of *Dpn*II enzyme (New England Biolabs, Beverly, MA) was electroporated in a Bio-Rad Gene Pulser (1 kV, 3 μF, expected τ: 0.6 to 1.1 ms). Cuvettes were left on ice for 10 mn then received 8 μl of 0.1 M $CaCl_2$/0.1 M $MgCl_2$, and each cuvette content was immediately placed in a 750 ml culture flask for a 15 mn incubation at 22–23°C. Forty ml of HL5 medium were added to each flask. The selection drug blasticidin (bsr) at 10 μg/ml was added 20–24 h later. Medium was changed 7 days later and selection proceeded for 3 more days.

Selection of bsr-resistant transformants for resistance to death

For these experiments, all media contained Penicillin-Streptomycin (Gibco BRL, Grand Island, NY), 100 units/ml and 100 μg/ml, respectively.

Populations of bsr-resistant transformants from 46 bsr-REMI independent electro-transfections were pooled two by two. Each pool was subjected to seven successive rounds of differentiation/re-growth (Cornillon *et al*, 1994). Briefly: bsr-REMI or untransformed control cells were led to differentiate in 50 ml Falcon flasks containing 0.8×10^6 cells in 2.5 ml of SB (Soerensen Buffer, starvation buffer) with cAMP 3 mM (Sigma Chemical Co, St Louis, MO) for 8 h. After a wash with 5 ml of SB, each flask received 2.5 ml of SB with 100 nM DIF (Affinity Research, Exeter, England) and were incubated for a further period of 24 h (Cornillon *et al*, 1994). HL5 (7.5 ml) plus 10 μg/ml blasticidin (or without blasticidin for untransformed control HMX44A cells) were added to each flask and surviving cells were allowed to re-grow for 40 h. Cells were then recovered, washed and counted. All the cells of each flask were then subjected to a second round of differentiation of 8+24 h without adjusting cell number. HL5 medium ($\pm$blasticidin) was then added as indicated previously and cells were allowed to re-grow again for 40 h. Five more rounds of differentiation/re-growth were done with a re-growth time of 64 h. Thus each of the 23 pools from the initially electroporated batches of cells was subjected to blasticidin selection and then to up to seven rounds of differentiation-regrowth.

Evaluation of *Dictyostelium* cell death

This was done in three different ways: (1) phase contrast microscopy: (2) fluorescein diacetate (FDA)/propidium iodide (PI) staining in fluorescence experiments, and (3) ratio after re-growth of [number of cells subjected to DIF in SB/number of cells subjected to SB alone].

Phase contrast microscopy Dying differentiating HMX44A cells showed prominent vacuolization, clearly visible already at 12 h in SB+DIF-1. Almost each cell rounded up and usually a single large vacuole progressively filled up most of the intracellular space. Cells subjected to SB alone, or cells with DIF in HL5 medium never showed vacuolization in our conditions.

Fluorescence staining A staining mix of fluorescein diacetate (FDA) and propidium iodide (PI) (Cornillon *et al*, 1994) can distinguish living cells (FDA+/PI−) from late dead cells (FDA−/PI+). Fluorescence microscopy experiments were done as previously described (Cornillon *et al*, 1994) with some modifications. Briefly: 1.6×10^6 cells per flask were led to differentiate once according to the protocol described above. After 12 or 24 h in the presence of DIF-1, FAD and PI (both from Sigma) stock solutions (10 mg/ml and 8×10^{-5} M, respectively) were added to final concentrations of 40 μg/ml and 2.5 μM, respectively. Flasks were incubated for 5 mn in the dark, washed once with 5 ml of SB, and cells were immediately observed using an inverted Axiovert 35 fluorescent microscope (C Zeiss, Oberkochen, Germany).

Cell death ratio after re-growth After 24 h of differentiation in SB$\pm$DIF, flasks received complete HL5 culture medium, and surviving cells were allowed to re-grow for various times. Proliferation of surviving cells was evaluated by hemocytometer counting under a phase contrast microscope and the ratio after re-growth of [cells subjected to DIF in SB/cells subjected to SB alone] was used as an index of cell death. This approach has the advantage of providing a quantitative estimate of cell death, but has the drawback of putative dependence on other parameters, such as variations of cell doubling time during re-growth.

Southern blot analysis

Genomic DNA was extracted according to (Chang *et al*, 1995) and digested with the indicated restriction enzymes. Digested DNA (2 μg) was fractionated by 0.8% agarose gel electrophoresis in 1 × TBE buffer without EtBr, EtBr stained, photographed, denatured for 30 mn in NaOH 0.5 N/NaCl 1.5 M, neutralized through two 15 mn incubations in Tris-HCl 0.5 M pH 7.5/NaCl 1.5 M and transferred to a Hybond-N membrane (Amersham Life Science, Buckinghamshire, England) overnight by capillary in high-salt buffer (20 × SSC). Each membrane was then baked at 80°C for 2 h and prehybridized in 6 × SSC/

2 × Denhart's/0.2% SDS for 2 h. Probes (see below) were random-labeled using ^{32}P-labeled dC and added to membranes at 1 to 1.5×10^6 c.p.m./ml of prehybridization buffer. After overnight hybridization at 65°C and three washes (3 × SSC/0.2% SDS, 1 × SSC/0.2% SDS and 0.5 × SSC/0.2% SDS), each for 30 mn at 65°C, membranes were autoradiographed at −80°C for 2 to 3 days. For cartography experiments, the whole of the pUCBsΔ*Bam*HI vector or only the blasticidin cassette were used as probes. The blasticidin cassette was extracted from the pUCBsrΔ*Bam*HI vector by *Hind*III+*Xba*I digestion and purified (GeneClean II Kit, BIO 101, La Jolla, CA) according to manufacturer's instructions.

Northern blot analysis

Cells (7×10^6/flask) were led to differentiate in 750 ml culture flask (Falcon). Two flasks were used per condition. Cells were recovered at various times of differentiation, pooled and washed with PBS, and RNA was extracted using TRIzol reagent (Gibco BRL) according to manufacturer's instructions. Total RNA (10 μg) was fractionated by 1.2% agarose gel electrophoresis in 1 × MOPS/3.7% formaldehyde, with 1 μl of 1 mg/ml EtBr added to each RNA sample prior to electrophoresis. Agarose gel was then soaked in 20 × SSC for 20 mn and RNA transferred to a Hybond-N membrane (Amersham Life Science), subsequently baked. Prehybridization was in formamide 50%/5 × SSPE/5 × Denhart's/0.1% SDS/300 μg/ml denatured salmon sperm DNA at 42°C for 3 to 4 h. Probes (see below) were added to membranes at 1.5 to 2×10^6 c.p.m./ml in formamide 50%/5 × SSPE/1 × Denhart's/0.1% SDS/300 μg/ml denatured salmon sperm DNA. After overnight hybridization at 42°C and two to three washes (2 × SSC/0.1% SDS twice and 1 × SSC/0.1 SDS) at 42°C for 20 mn, membranes were autoradiographed at −80°C for 2 days. Membranes were stripped for 2 h in a 5 mM Tris-HCL pH 8.0/2 mM EDTA/0.1 × Denhart's solution at 65°C. *ecmA* and *ecmB* cDNAs, obtained from JG Williams, University College of London, *lg7* cDNA obtained from JD Gross, University of Oxford, and the 'right flank' C5 PCR product were successively used as probes.

PCR to isolate C5 genomic flanking sequences

C5 genomic DNA (60 μg) was *Xba*I digested and fractionated by 0.8% LMP agarose gel electrophoresis in 1 × TAE buffer without EtBr. After EtBr staining of the molecular weight lane, an agarose band of about 0.5 cm corresponding to the size obtained by Southern blot for the *Xba*I digested DNA with the blasticidin cassette as a probe (not shown) was recovered from the *Xba*I digested genomic DNA lane. DNA was recovered by melting of the agarose at 65°C, phenol/chloroform extraction and ethanol precipitation. An aliquot of DNA was tested back by Southern blot to check for the presence of the *Xba*I DNA fragment containing the blasticidin cassette. About 40 ng of the digested DNA thus obtained was ligated on itself in a 15 μl ligation reaction with 200 U of ligase (New England Biolabs) for 16 h. A fraction of the ligation reaction (2 μl) was used in a 40 μl PCR reaction with 4 U of Taq Polymerase (Gibco BRL) and 40 pmol of each of the oligonucleotides b8 5′-GGGGAAATGTGCGCGG-3′ and b5 5′-GCCGCTCCCACATGATG-3′ as primers (see Figure 1C). For this and further PCR reactions, conditions were 30 cycles of 45 s at 94°C, 30 s at 52°C and 2.5 min at 72°C, and the PCR product was analyzed by gel electrophoresis. The band was picked once with a toothpick and the extremity of the toothpick was dipped in 20 μl of sterile MilliQ water. One μl of water containing eluted DNA was used in a second PCR reaction with the 'internal' b7 5′-CGCTCATGAGACAATAACC-3′ and b1 5′-CTCTGTCGCTACTTCTAC-3′ primers. For verification purposes, 100 nanograms of total genomic DNA from C5 and HMX44A, or 10 ng of the right flank PCR product obtained above were used as template in PCR reaction using b7 and b9 5′-AATCGCTGTTACACTGCAG-3′ primers, or b2+b9 primers. b9 was designed according to the preliminary sequence (Genome Express, Lyon, France) of the right flank PCR product.

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