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Use of a transactive regulatory mutant of *Dictyostelium discoideum* in a eucaryotic expression system

Birgit W.Wetterauer, Klaus Salger and Harry K.MacWilliams
Zoologisches Institut der Ludwig-Maximilians Universität München, Luisenstraße 14, 8000 München 2, Germany

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ABSTRACT

The discoidin proteins of *Dictyostelium discoideum* are highly expressed during development. The Disc Iγ promoter allows the regulation of heterologous protein expression by experimental conditions (1). We report conditions under which the promoter activity is efficiently repressed during growth in the wildtype strain AX2. In addition we show that a mutant which overexpresses the discoidins also overexpresses the reporter genes β-galactosidase, luciferase and CAT 10- to 100-fold when these are placed under the control of a Disc Iγ promoter. This system may be generally useful for the overexpression of genes in *Dictyostelium*, both for functional studies *in vivo* and for the production of heterologous proteins for purification.

INTRODUCTION

Highly active promoters are important tools in gene technology. In *Dictyostelium* most commonly-used constructs employ actin promoters. These have been used to drive expression of drug resistance genes in selection cassettes (2), for expression of antisense RNA (3) and overexpression of native or modified *Dictyostelium* genes (4, 5).

Several genes other than the actins are highly expressed in *Dictyostelium discoideum*. Among these are the genes encoded by the discoidin multigene family (6, 7). The discoidin promoters could be of particular interest for expression of genes transformed into *Dictyostelium* because they can be turned on and off by relatively simple experimental manipulations. This flexibility arises from the normal regulation of the discoidin promoter during the *Dictyostelium* life cycle. There is no discoidin expression in early growth phase; the promoter becomes activated by an extracellular factor, PSF, which accumulates as cell density increases (8).

Well established transformation protocols in *Dictyostelium* rely on strains which are able to grow in synthetic media. In such media the discoidin promoters are active even at very low cell densities. However, it has been reported that the activity of the promoter during axenic growth can be reduced by addition of folate and thereafter be induced by starvation (1). This could make discoidin promoters particularly useful.

We previously isolated an axenically growing mutant (VI88), in which the discoidin genes are overexpressed at the mRNA level. Stemming from an initial interest in signal transduction in VI88 we investigated whether the mutation acts in trans; this is indeed the case. Our results suggest that the discoidin promoter in VI88 could be generally useful for high level expression of trans genes, either for the modification of cellular functions or for the production of proteins for *in vitro* experiments.

MATERIALS AND METHODS

The strains AX2 and VI88, which is a derivative of AX2 (12), were used in the experiments as indicated. The cells were either grown in axenic culture (21) or in suspension culture with *Enterobacter aerogenes* in 50mM potassium phosphate buffer, pH 6.2 (KK_2).

Cells were stably transformed according to the standard procedure with $CaCl_2$ (2). Three different reporter constructs were used, pDPLac/Neo, pVEDisLuc, and pAVDisCAT-411. They are used to express the β-galactosidase, the luciferase (12) and CAT (17) under the control of the discoidin Iγ promoter. pDPLac/Neo and pVEDisLuc contain the entire Disc Iγ promoter, while in pAVDisCAT-411 only a fragment of the promoter extending 411 bp upstream the ATG is used (convenient vectors for other constructs are proposed in (1)). Transformants in axenic culture were grown in the presence of 20 μg G418; in bacterial culture 50 μg G418 were added and cells were diluted in fresh bacterial suspension every day. VI88 scarcely grows beyond 5×10^6 cell/ml, while AX2 reaches about twice this density. VI88 cells are considerably larger, however, so that the final mass is comparable in mutant and parental strains. We have also observed that generation time of VI88 in axenic medium is about twice that of AX2.

The luciferase assays were performed with a Berthold luminometer as described (12, 22). For CAT assays the liquid scintillation method was used (23). β-Gal detection on colony-blot used the standard protocol of fixation and detection (24) after transfer of colonies growing on agar plates onto the nitrocellulose. (13). Detection of β-Gal activity of lysed cells with methyl-umbelliferyl-galactoside was performed as described (24). Determination of protein concentrations were done with amido black (25) and enzyme activities are indicated per μg protein.

For Westerns blots the monoclonal antibody 80–52–13 against discoidin I was used in standard protocols using TBS with 0.05% Tween and 3% BSA for incubation and TBS with 0.05%

Tween for washes (12). The secondary antibody (Promega, goat anti mouse IgG), labelled with horse radish peroxidase, was detected with chloronaphtol and H_2O_2. RNA preparations and Northern blots are described in (26). The blots were hybridized with the 350 bp EcoRI/KpnI fragment from the coding region of Disc Iγ, which had been labelled by random priming.

RESULTS

Discoidin expression in AX2

The expression of the discoidins has been well characterized for the wildtype strain NC4 (8). This strain feeds on bacteria and is unable to grow in axenic media. There is no expression of the discoidins until the cells reach a densitiy of about 1×10^6. The cells continuously secrete a factor, PSF, which accumulates proportionally to the cell density and gradually induces the discoidins and some other genes (9). A second factor, CMF, also induces discoidin expression (10); the discoidins are repressed by extracellular cAMP (6) and folate (11).

In order to investigate expression patterns in the axenic strain AX2, discoidin induction was monitored using luciferase as a reporter gene under the control of the complete (1.2 kb) Disc Iγ promoter (pVEDisLuc). Luciferase activity faithfully reflected the induction of the discoidin proteins. The expression pattern was the same as described for NC4 (Fig. 1) in the buffer used by Clarke and coworkers (20 mM Na_2K phosphate buffer pH 6.2) . We found, however that in KK_2-buffer (50 mM potassium phosphate, pH 6.2) Disc Iγ promoter activity is delayed until late growth phase. Further experiments showed that the molarity is not important, and that only the salt composition of the buffer determines the onset of discoidin expression (data not shown). The final levels of discoidin protein and luciferase in stationary phase are the same in both buffers. We conclude that the regulation of discoidins during growth on bacteria is comparable in NC4 and AX2, and that the onset of discoidin expression can easily be determined by a simple choice of buffer conditions. For all subsequent experiments cells were grown in potassium phosphate buffer.

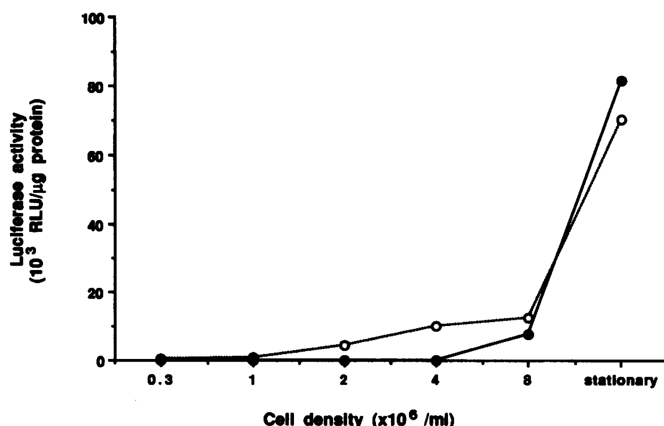

Figure 1. Buffer conditions determine the onset of discoidin expression during bacterial growth. AX2 cells transformed with pVEDsLuc were grown in bacterial suspension in 20 mM K/Na_2-phosphate buffer (○) and in 50 mM KK_2-buffer (●). In Na/K buffer the discoidin promotor becomes active at moderate cell densities. In KK_2 buffer significant promotor activities are seen only when high cell desnities are reached.

Discoidin overexpression in the mutant VI88

We recently identified a discoidin-overexpressing mutant (VI88) by immunoscreening of colony blots (12). Discoidin appears to be (a) overexpressed and (b) prematurely expressed in VI88. The differerence in expression levels is already apparent in total cell extracts analysed on coomassie stained denaturing PAGE gels (Fig. 2A). AX2 and VI88 were harvested at different densities during growth in a bacteria suspension. In VI88 a prominent 30 kDa band was seen; this band was not detectable with coomassie stain in AX2. The prominent 30kd band in VI88 comigrates with discoidin I immunoractivity and the difference in temporal pattern and quantity of discoidin expression can be confirmed by examination on Western blots (12).

Discoidin expression in transformed strains

The most frequently used vectors in *Dictyostelium* carry actin promoter-based neomycin-resistance cassettes. These vectors typically integrate into the genome in about 100 copies. The introduction of multiple copies of a promoter might lead to competition for specific factors necessary for transcription. To determine whether this phenomenon affects discoidin reporter constructs, we compared the expression of the endogenous discoidin genes in pVEDisLuc transformed and untransformed AX2 and VI88 (Fig. 3). The time course of induction is not altered by the transformation in either AX2 or VI88. The levels of expression, however, seem to be changed. In both strains transformation seems to increase transcript levels in late growth phase rather than to decrease them. The difference was apparent on Northern but not on Western blots, presumably due to the

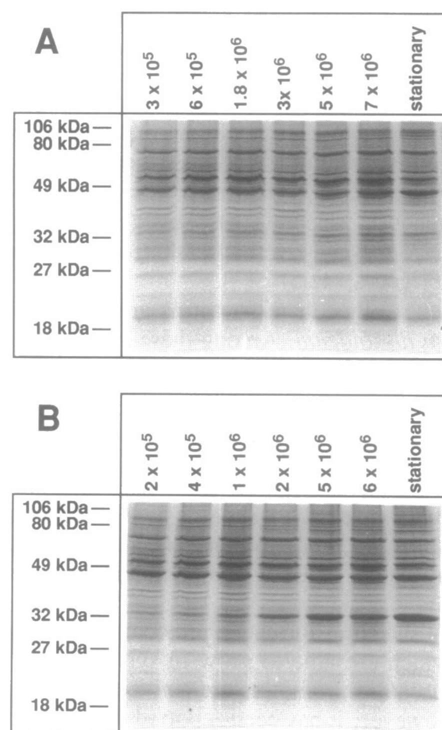

Figure 2. Overexpression of the discoidin proteins in VI88. AX2 (A) and VI88 (B) cells were grown in a bacterial suspension in KK_2-buffer. 20 μg of total extracts from cells at different densities were separated on 12% polyacrylamid gels and stained with coomassie blue.

indirect detection system used for discoidin protein. To quantifity the effect, we measured discoidin protein levels in serial dilutions using slot blots; several independent preparations of stationary AX2, AX2DisLuc, VI88 and VI88 DisLuc were compared. Discodin protein levels were about 10-fold higher in VI88 than in AX2. Transformed VI88 and AX2 had roughly 2-fold higher protein levels than the untransformed parents. We conclude that the activity of the discoidin promoter in the transformed strains is not negatively influenced by competition for factors necessary for transcription. The results instead suggest that a negative regulator might become limiting in the the transformed strains.

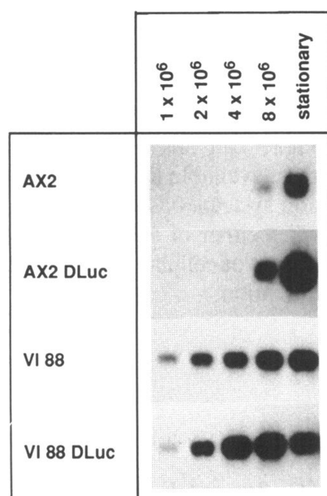

Figure 3. Transformation enhances endogenous discoidin expression. AX2, AX2DisLuc, VI88 and VI88DisLuc were grown in bacterial suspension in 50 mM KK_2 buffer to the indicated cell densities and harvested for RNA extraction. Northern blots were hybridized with the EcoRI/KpnI fragment of the coding part of the discoidin Iγ gene. Equal loading was verified by staining parallel gels with ethidium bromide. The RNAs were treated in parallel, AX2 and AX2DisLuc were exposed for 3 days, VI88 and VI88DisLuc were exposed overnight.

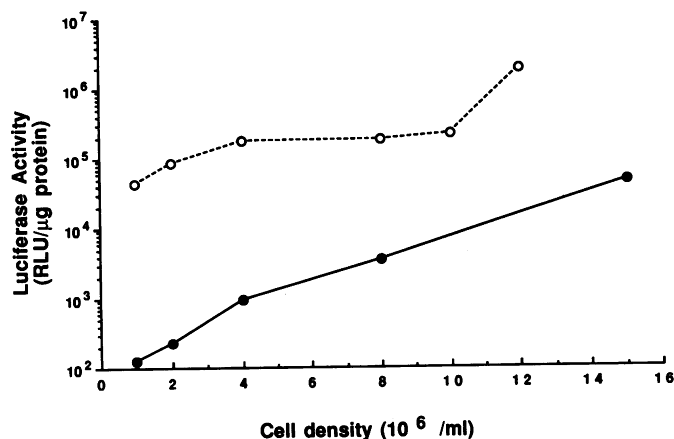

Figure 4. Overexpression of the luciferase reporter in VI88 during growth on bacteria. AX2 and VI88 cells transformed with pVEDisLuc were grown in a bacterial suspension in 50 mM KK_2-buffer. Cells at different densities were extracted for determination of the luciferase activies as described in (22). Luciferase activity in VI88 DLuc (○) is about 100-fold higher than in AX2DLuc (●)

Reporter expression in the mutant VI88

VI88 and the parental strain AX2 were transformed with the discoidin-luciferase vector (pVEDisLuc) and the luciferase expression was compared during growth on bacteria in 50 mM KK_2. In the mutant, luciferase expression was already detectable at cell densities below 1×10^6 (Fig. 4), as was discoidin protein (Fig. 2B). At this cell density, luciferase activity in AX2 was not significantly above background levels. At higher densities, where discoidin induction occured in the parent, the activity in VI88 was 10- to 100-fold higher. Thus two aspects of the VI88 phenotype, overexpression and precocious expression of discoidin, are both reflected in the activity of the luciferase reporter.

The VI88 phenotype can also be seen in strains transformed with a discoidin-β-galactosidase vector. AX2 and VI88 transformed with pDPLac/Neo were inoculated on SM/2 agar plates with *Enterobacter aerogenes* and grown for 3 days. The colonies were transfered to nitrocellulose, fixed, and stained for β-galactosidase activity at room temperature (13) (Fig. 5). In order to obtain comparable staining intensities in the aggregating cells, AX2 was stained longer than VI88 (5h versus 15 min); this shows that the maximal expression levels in VI88 are higher. Even with the staining reaction adjusted to compensate for the difference in activity level, a difference in the distribution of staining remained visible: VI88 has considerable activity in the growth zone, while AX2 has none. These results confirm those obtained with the luciferase reporter: the overexpression and the premature expression of discoidins are reproduced in the β-gal reporter construct.

Axenic culture of *Dictyostelium* is more convenient than growth on bacteria if large quanitities are needed, for example for biochemical experiments. We therefore compared the expression of reporter contructs in AX2 and VI88 during axenic growth (Fig. 6). Cells were harvested at 3×10^6 and enzyme activities detected as previously. All three reporter constructs (luciferase, CAT and β-galactosidase) are overexpressed about 30-fold in VI88.

DISCUSSION

For high level expression in *Dictyostelium* highly active promoters are needed. A number of these have been used to drive the expression of genes in transformed strains; these include the promoters of the V18 gene (14), the actin-6 and actin-15 (2), discoidin Iγ (15) and sp60 (16). The latter promoter is active only in late development and is therefore inconvenient for handling of large quantities of cells needed for purification of proteins. V18, the actins and discoidin are all expressed in growth or early development, i.e. at stages which are easily obtainable in large quantities. Of these only the discoidins offer the possiblity of growing cells in the absence of promoter activity.

For production of proteins the maximal activity of the promoter is important. Although direct quantitative comparisions have not been made, results of β-galactosidase staining reactions and data from Nellen and coworkers (17) suggest that the activities of the V18, discoidin and actin promoters are comparable in wildtype cells. In VI88, however, the discoidin promoter is overexpressed by a factor of 10 to 100; thus the discoidin/VI88 combination appears to offer the highest promoter activity currently available in *Dictyostelium*.

The protein levels for the reporter products β-galactosidase, luciferase and CAT, although increased 10- to 100-fold when compared to AX2, do not approach those seen for the endogenous discoidins. Possible reasons may be protein stability and translational efficiency. There is probably not a major difference in protein stability: both the discoidins and the reporters β-galactosidase (MacWilliams, unpublished), CAT (Nellen, personal communication) and luciferase (16) are stable in *Dictyostelium*. It thus appears likely that the difference in protein levels between the discoidins and the reporter products is due to different translational efficiency. The *Dictyostelium* genome is extremely rich in A and T; in the coding portions of genes this is reflected by a strong preference for codons with A or T in degenerate positions (18) and there is evidence that codon usage influences transcriptional efficiency in *Dictyostelium* (19). It may be necessary to take this into account in designing protein expression systems in *Dictyostelium*, whether they are based on the discoidin promoter or other promoters.

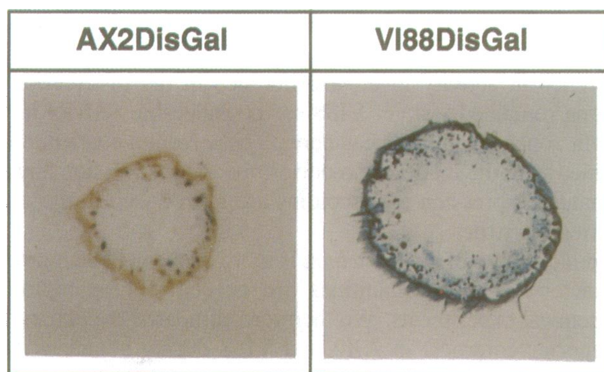

Figure 5. Precocious expression of β-galactosidase. AX2 and VI88 cells transformed with pDPLac/Neo were grown on a solid surface with *Enterobacter aerogenes*. Colonies were transfered to nitrocellulose and stained for β-galactosidase activity at room temperature as described in (24). AX2 was stained for 5h, VI88 for 15 min.

Relative to maximal expression levels, the expression of discoidin in VI88 is low when cells are growing on bacteria. We have, however, not seen VI88 cells which are without any discoidin promoter activity. This could make the strain unsuitable for expression of toxic compounds. In such cases it may well be possible to use discoidin-promoter based vectors in AX2 cells. Although the promoter is active in normal axenic growth, its activity can be reversibly reduced by addition of folate (1) (The folate repression can be seen in VI88 as well but reporter activity after folate treatment is still significantly above the levels of untreated AX2, unpublished). If this repression is not sufficient, one can grow AX2 cells bacterially on KK_2 buffer. Under these conditions, discoidin promoter activity is extremely low until shortly before stationary phase. Recent results suggest that transformations can also be carried out on bacteria, where no discoidin expression is detectable (20 and MacWilliams unpublished).

Through the choice of host and growth conditions, the activity of the discoidin promoter can thus be varied from essentially zero to probably the highest available in *Dictyostelium* with the same construct. Expression systems based on this promoter, promise to be useful for studies either of cellular functions by antisense RNA and overexpression of cellular regulators, or for expression of proteins for purification.

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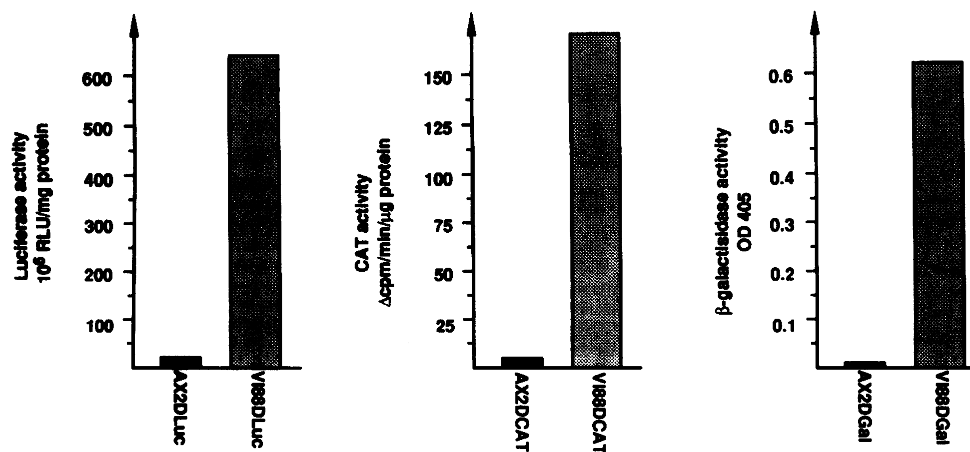

Figure 6. Overexpression of different reporter proteins in axenic growth. AX2 and VI88 transformed with different reporter constructs were harvested at 3×10^6 cells/ml after growth in axenic medium. Comparison of the reporter activies shows that all three reporters are overexpressed about 30-fold in VI88.

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