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Two nuclear DNA binding proteins of *Dictyostelium discoideum* with a high affinity for poly(dA)-poly(dT)

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SUMMARY

Intergenic regions of the Dictyostelium genome contain an extremely high proportion of AT base pairs. Those intergenic regions which have been subjected to nucleotide sequence analysis are predominantly composed of alternating runs of poly(dA) and poly(dT) and there is evidence to suggest that nucleosomes do not form on such sequences. We have identified two nuclear proteins, of molecular weight 70,000 and 74,000 daltons, which bind only to intergenic regions of a cloned Dictyostelium gene. Binding is specifically inhibited in the presence of synthetic poly(dA) - poly(dT) as competitor. These proteins may play some role in the chromosomal organization of intergenic regions in Dictyostelium discoideum.

INTRODUCTION

The discoidin 1 proteins are carbohydrate binding proteins of unknown function which constitute over 1% of the cellular protein in Dictyostelium cells after aggregation. There are three discoidin 1 proteins (1a, 1b and 1c) which are the products of different genes (1,2).Changes in the concentration of discoidin 1 mRNA are brought about by changes in the rate of discoidin 1 gene transcription (3). The cessation of discoidin 1 transcription occurs at a time during development when the intracellular concentration of cAMP is increasing and addition of cAMP to cells which are expressing the discoidin 1 gene causes a very rapid and specific drop in the rate of discoidin gene transcription (4). Because it is a simple matter to isolate large numbers of Dictyostelium cells which are at a defined stage in differentiation, this is an excellent system in which to search for proteins which interact with genes to control their rate of transcription.

There are now a number of instances in which eukaryotic DNA binding proteins that interact with specific genes have been identified. Thus the T antigen of SV40 has been shown to have specific binding sites on SV40 DNA (5) and binding has been shown to affect transcription in an in vitro

system (6,7,8). Engelke et al. (8) have identified the binding site within the gene of a protein required for transcription of the gene encoding the 5s RNA of Xenopus oocytes. Activated glucocorticoid receptor has been shown to bind to the terminal repeats of mammary tumour virus DNA (9) and progesterone receptor complex has been shown to bind to specific sequences upstream of several hormone responsive genes (10). Also there are a number of instances where proteins with as yet unidentified function have been shown to bind to specific regions of cloned DNA sequences. These include the Drosophila protein DB2 which binds to cloned Drosophila DNA segments of unknown function (11), a Drosophila protein which binds upstream of the heat shock gene (12) and nuclear proteins which bind upstream of the chicken lysozyme gene (13). Finally there are proteins such as the D1 protein of Drosophila (14) which show a general affinity for AT rich DNA.

We have used in situ transfer and DNA binding (15,16) to identify proteins which interact with a cloned discoidin 1 gene. We have identified two proteins, BP1 and BP2, which bind to specific restriction fragments derived from upstream of the discoidin 1 gene. These do not, however, appear to be specifically involved in controlling discoidin 1 gene expression, rather they appear to be binding proteins with a highly specific affinity for A-T rich DNA.

MATERIALS AND METHODS

Phenyl methyl sulfonyl fluoride (PMSF), Nα tosyl chloromethylketone bovine serum albumin Fraction V and Polyvinylpyrrolidone 40, were purchased from Sigma, Ficoll 400 was obtained from Pharmacia, Agarose (low electroendosmosis grade) restriction enzymes and T4 DNA polymerase were from Bethesda Research Laboratories and nitrocellulose filters SM1136 were from Sartorius. Electrophoresis chemicals were from BDH. All the other reagents were analytical grade.

Cell culture and development

Axenic cells (Strain Ax2 ATCC2439 from J. Ashworth) were grown in liquid medium and harvested when the culture reached a density of 2×10^6 cells/ml. The strain V12M2 was grown on SM agar plates in the presence of Klebsiella aerogenes. For both strains development was performed at a cell density of 1×10^7 cells/ml in KK2 medium (20mM potassium phosphate, 2mM $MgSO_4$ pH 6.2) at 22°C on a gyratory shaker at 120 rpm.

Extraction and preparation of nuclei

At different times after initiation of development aliquots were

removed, cells were collected by centrifugation and they were either lysed immediately (whole cell extracts) or used for preparation of nuclei. Whole cell extracts were prepared by boiling samples for 3 minutes in sample buffer: 62.5mM Tris pH 6.8 2% SDS, 5% 2-mercaptoethanol, 10% glycerol.

Nuclei were prepared using a modification of the method described by Jacobson (17). All buffers contained 50µg/ml phenyl methyl sulfonyl fluoride PMSF and 0.1mM Nα tosyl chloromethylketone. Cells were lysed by vortexing in lysis buffer (50mM HEPES pH 7.5, 5mM magnesium acetate, 10% sucrose), containing 2% Cemulsol NPT 12. Nuclei were collected by centrifugation for 10 min at 5,800 g. This supernatant constitutes the cytoplasmic fraction. Nuclei were resuspended in lysis buffer containing 2% Cemulsol NPT 12 and centrifuged for 10 min at 5,800 g. Nuclei were then resuspended in lysis buffer containing only 0.1% Cemulsol and centrifuged for 10 min at 5,800 g. The nuclear pellet was resuspended and boiled for three minutes in Laemmli sample buffer. The cytoplasmic fraction, containing 2% Cemulsol, was made to 10% SDS, 62.5mM Tris pH 6.8, 5% mercaptoethanol, 10% glycerol and boiled for 3 minutes.

Electrophoresis and transfer

Extracts were loaded onto polyacrylamide-SDS gels prepared according to Laemmli (18). Separating gels (16cm height) contained 10% acrylamide (with a bis-acrylamide to acrylamide ratio of 0.8 to 30) and 6M urea. After electrophoresis, proteins were transferred to nitrocellulose filters (Sartorius SM 11306) by the electrophoretic procedure of Towbin et al. (19). Transfer was for 20 hours at room temperature at a constant current of 100 mA.

Binding to DNA

After transfer, nitrocellulose sheets were soaked with gentle agitation in binding buffer: 10mM Tris HCl pH 7.0 50mM NaCl, 2mM EDTA, 0.1mM dithiothreitol 0.02% bovine serum albumin, 0.02% polyvinylpyrolidone 40, 0.02% Ficoll 400 (16), for several hours at room temperature with one change of buffer. They were then incubated for one hour at room temperature in sealed plastic bags containing 10ml of ^{32}P-labelled DNA, at >10^5 cpm/ml in binding buffer. DNA was removed and filters were rinsed for one hour with two changes of buffer containing 150mM NaCl. They were finally exposed for autoradiography at -70°C with an intensifying screen. Areas on the nitrocellulose filters showing DNA binding were excised, the DNA was eluted in the presence of 2% SDS, and analysed on a 0.8% agarose gel according to Jack et al. (29). The agarose gel was dried under vacuum and exposed for

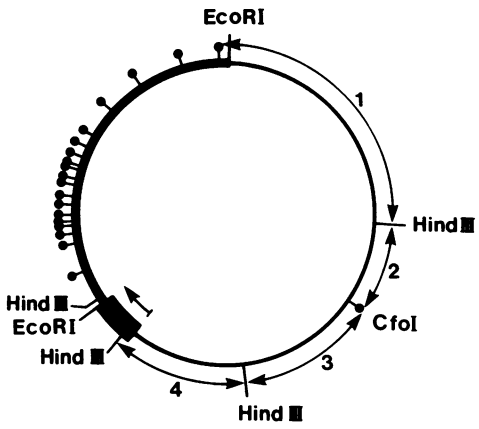

Fig. 1
Restriction map of pDd17

The plasmid PDd17 contains a 7Kb fragment of Dictyostelium DNA inserted into the EcoR1 site of the pBR322 derivative pAT153. The cloned Dictyostelium fragment contains the 5' half of the discoidin 1c gene (21). The discoidin gene sequences are represented by the thickest line, the direction of transcription being indicated by an arrow inside the circle. Sequences upstream of the discoidin 1c gene are represented by the thinnest line, the region being subdivided into four segments based upon the cleavage positions of Hind III and Cfol. Vector sequences are represented by a line of intermediate thickness and the multiple cleavage sites for Cfol are indicated only by a symbol (↑).

autoradiography.

Preparation of radioactive 17 DNA

pDd17 DNA was digested using Cfol and Hind III according to manufacturers' instructions (Bethesda Research Laboratories). The DNA was labelled with T4 DNA polymerase in the presence of α ^{32}P-dCTP (Amersham International, 3 Ci/mmole) (20). Conditions were adjusted such as to replace 100 nucleotides at the terminus of each fragment yielding DNA with a specific activity of about $4x10^6$ cpm/µg DNA. The labelled DNA was analysed by electrophoresis on a 0.8% agarose gel in 88mM Tris, 89mM boric acid, 2.5mM Na EDTA pH 8.2.

RESULTS

The proteins BP1 and BP2 bind to restriction fragments derived from the discoidin 1c gene

Total cellular proteins isolated from Dictyostelium amoebae at different stages of early development were separated by SDS acrylamide gel

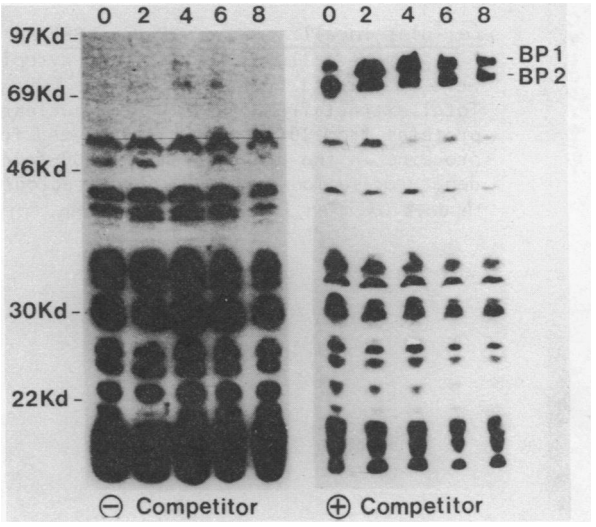

Fig. 2
Total cellular DNA binding proteins analyzed at different stages of development

Total cell extracts prepared from V12M2 cells at times of development up to 8 hours were analyzed as described in the Methods section. Each contains the protein from 2.6×10^6 cells and the time of development at which the cells were harvested is indicated above each lane. Two parallel binding reactions were performed, one in the absence of competitor DNA and one in the presence of a 3,000-fold weight excess over probe of sonicated heat denatured salmon sperm DNA. The probe was at 2×10^7 cmp/µg and autoradiography was for 16 hr with an intensifying screen.

electrophoresis, transferred electrophoretically to nitrocellulose filters and their binding to restriction fragments derived from the plasmid pDd17 was determined. This plasmid contains the 5' half of the discoidin 1c gene and 7kb of upstream flanking sequence inserted into the EcoR1 site of pAT153 (21). Digestion with Hind III and Cfol cleaves the Dictyostelium DNA insert to yield five fragments and cleaves the plasmid DNA to yield a large number of very small fragments (Fig. 1). This mixture of DNA fragments was isotopically labelled by replacement synthesis with T4 DNA polymerase and incubated with the nitrocellulose filters. Many proteins bound labelled DNA (Fig. 2) but in a large number of experiments there were no reproducible developmental changes in the spectrum of binding proteins. Many basic proteins, in addition to the histones, act as DNA binding proteins, Therefore, in order to identify proteins which display specific binding, we performed parallel binding analyses in the presence of a large

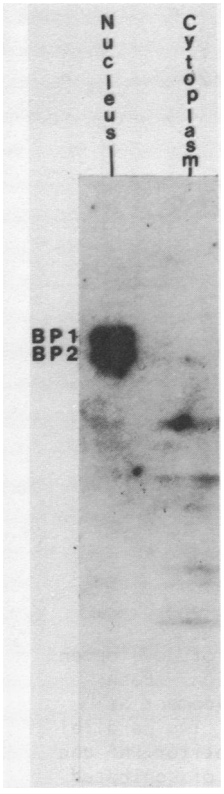

Fig.3
Cellular localization of BP1 and BP2

Total cellular, nuclear and cytoplasmic extracts were prepared from V12M2 cells at 4 hr of development. Total extract from 10^6 cells and nuclear and cytoplasmic proteins from 10^7 cells were analyzed for DNA binding proteins in the presence of a 3,000-fold excess of denatured salmon sperm DNA. Autoradiography was for 14 days with an intensifying screen.

excess of salmon sperm competitor DNA. Using native salmon sperm DNA, at up to a 10,000 fold weight excess, there was a uniform reduction in binding of the probe to the various resolved polypeptides (results not shown). However, when heat denatured salmon sperm DNA was used a quite different result was obtained. This competitor drastically reduced the number of proteins which bound labelled DNA but two proteins, of apparent molecular weight 70,000 and 74,000 daltons, were almost totally resistant to competition (Fig. 2). We term the 74,000 dalton protein BP1, and the 70,000 dalton protein BP2. The competition of binding to the other proteins was somewhat variable such that in some cases the only detectable binding was to BP1 and BP2 (e.g. compare 2 with Fig. 3). An unexpected but totally reproducible, feature of these experiments was that the amount of labelled DNA bound to these proteins actually increased by a factor of up to ten fold when competitor was present. This was unexpected because only a tiny fraction of the probe binds to the proteins even in the absence of competitor. Hence,

in principle at least, the increase observed ought not to be the result of an increased probe availability. (NB This may indicate that the actual kinetics of binding are similar to those observed in colony hybridization where, despite the presence of a large total excess of probe, the hybridization is driven by the effective DNA excess present in the local area of the bacterial colony (22).

BP1 and BP2 are nuclear proteins

We chose initially to analyze total cellular proteins in order that we might not miss some potentially important regulatory protein, either because of a technical artefact such as proteolysis during nuclear isolation or because such proteins might be predominantly located in the cytoplasm prior to activation by a second messenger such as cAMP. Having identified BP1 and BP2 as proteins which showed selective binding to pDd17 we wished to establish their cellular location. Amoebae at four hours of development were lysed with detergent and separated into a nuclear and cytoplasmic fraction. Most of the DNA binding proteins were in the nuclear fraction but there were a number of cytoplasmic DNA binding proteins. These proved useful as they indicated that the separation of nucleus and cytoplasm was very effective - there being very little of the cytoplasmic DNA binding proteins in the nuclear fraction and vice versa. The BP1 and BP2 polypeptides were totally nuclear confined and the results of a typical experiment, performed in the presence of salmon sperm competitor DNA, are shown in Fig. 3.

BP1 and BP2 display selective binding to three restriction fragments derived from upstream of discoidin 1 gene

Segments of filters bearing the BP1 and BP2 polypeptides were identified by alignment with the autoradiogram and excised separately. The labelled restriction fragments were eluted by denaturing the proteins with SDS and analyzed by agarose gel electrophoresis. Only three of the five Dictyostelium derived restriction fragments were bound to BP1 and BP2 (Fig. 4). One of the fragments which was not bound (fragment 2, see Fig. 1) is a Hind III-Cfol fragment located 3.5Kb upstream of the gene. The small fragment containing the bulk of the coding region of the discoidin 1c gene and all of the small Cfol fragments derived from pAT153 were also not bound. These results derived from an experiment where the binding was performed in the presence of denatured salmon sperm competitor DNA but identical results were obtained when competitor DNA was not present. Restriction fragments were purified from one of the DNA binding polypeptides which was sensitive

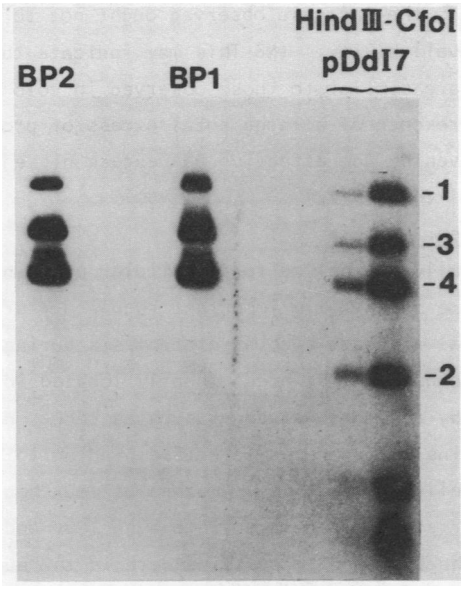

Fig.4
Determination of the restriction fragments bound by BP1 and BP2

Labelled restriction fragments bound to BP1 and BP2 were recovered by elution with SDS containing buffer and analyzed by agarose gel electrophoresis. In this particular experiment electro-blotting was performed using total cellular protein isolated from cells at 4 hr of development and there was a 3,000-fold weight excess of denatured salmon sperm competitor DNA in the binding reaction. Two different amounts ($2x10^3$ cpm and $2x10^4$ cpm) of the pDd17 probe were also analyzed on the same gel.

Cleavage of pDd17 with Hind III and Cfol yields four large fragments derived from upstream of the discoidin 1c gene (Fig. 1) and each of these fragments is identified at the extreme right of the figure. The small fragment containing the bulk of the coding sequence of the discoidin gene and the many small fragments derived from the vector are not well resolved on this gel. In heavily exposed autoradiograms such as this the DNA fragments bound to and eluted from BP1 and BP2 migrate as somewhat broader bands than the control fragments, presumably because of degradation.

to inhibition by competitor DNA and there was no evidence of selectivity of binding (data not shown). This indicates that the selectivity seen with BP1 and BP2 is not the result of an artefact of the analysis procedure.

DNA binding to BP1 and BP2 is strongly inhibited by competition with poly(dA) and poly(dT)

The three DNA fragments which were bound by BP1 and BP2 derive from intergenic DNA. These regions of the Dictyostelium genome are extremely rich in A-T base pairs with a major portion of the DNA being

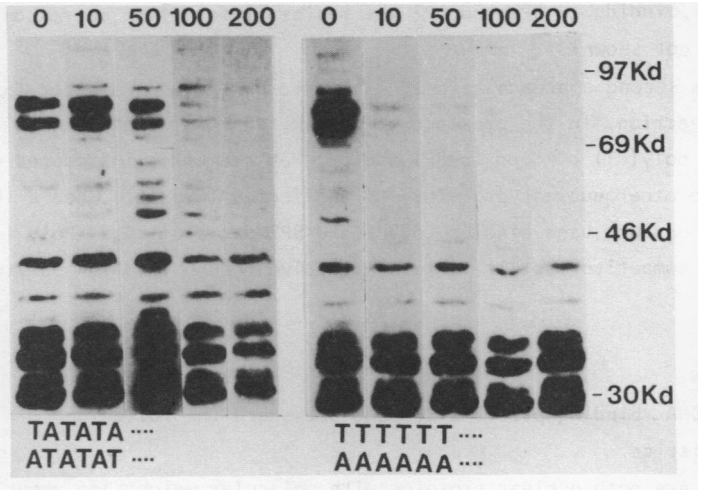

Fig.5
The effect of synthetic dA-dT sequences on DNA binding by BP1 and BP2

Nuclear extract prepared from V12M2 cells at 4 hr of development was subjected to electrophoresis on a 10% acrylamide gel. Each lane of the gel contained an identical amount of sample and after electro-blotting to nitrocellulose the sheet was cut into ten identical strips. The strips were incubated separately with varying amounts of the indicated competitor DNA. (The samples at the left were incubated with poly(dA.dT) and those at the right with poly(dA).poly(dT). The weight excess of competitor over probe is indicated above each lane. After binding and washing the nitrocellulose strips were re-aligned for autoradiography for 16 hr with an intensifying screen.

composed of alternating tracts of poly(dA) and poly(dT). Also, we have determined the base composition of fragment 2, the fragment which is not bound by BP1 and BP2, and we find that it has an unusually high GC content of 56% (Karran and Garreau, unpublished). We wished to determine therefore whether BP1 and BP2 were displaying specific binding to the discoidin 1c gene or whether they were generalized dA-dT binding proteins. This question was approached in two ways. Firstly we performed a binding experiment identical to those described above except that we used a cloned Dictyostelium actin gene. This recombinant Eco 13.4.9 is an independent isolate of the well characterized actin M6 gene (24). The clone contains the entire actin M6 gene flanked by 1.2Kb of AT rich DNA at the 5' end (24) and 1.8Kb at the 3' end and inserted into the EcoR1 site of PMB9. The DNA was labelled after cleavage with Hind III which cleaves once within the actin structural gene and once within PBM9. In the absence of competitor, exactly the same pattern of DNA binding proteins observed with the discoidin 1c gene was obtained and

again only binding to BP1 and BP2 was resistant to salmon sperm competitor DNA (data not shown).

As a second approach we used synthetic DNA as a competitor in the binding reaction. In the presence of only a ten fold weight excess of poly(dA), poly(dT) binding to BP1 and BP2 was dramatically reduced while binding to other nuclear proteins was unaffected (Fig. 5). When a 100-fold weight excess was used binding to BP1 and BP2 was abolished. Poly (dA.dT) is also a competitor but it is less effective than poly(dA) - poly(dT) (Fig. 5).

DISCUSSION

The DNA binding proteins BP1 and BP2 share the following characteristics.

They are both nuclear proteins with molecular weights of about 70,000 daltons. They bind the same three restriction fragments from the flanking region of the discoidin 1c gene and their binding to DNA displays identical relative sensitivities to different competitor DNA sequences. The most obvious conclusion would be that BP2 is a degradation product of BP1. However, if this is the case, then it seems likely that the proteolysis must occur *in vivo* because we detect both proteins in total extracts prepared by solubilizing whole cells in SDS buffer.

Both proteins are highly specific in their binding to AT rich DNA. They bind only three AT rich DNA fragments from upstream of the discoidin 1c gene and the only upstream fragment (fragment 2, Fig. 1) which is not bound has a much lower AT content. (NB Given the usually high AT content of intergenic regions of the *Dictyostelium* genome this may indicate that fragment 2 contains another structural gene - the distance between the discoidin 1c gene and this upstream sequence being about that expected for the average intergenic region in *Dictyostelium*. This possibility is currently being investigated). Also, binding to BP1 and BP2 is almost totally resistant to competition with denatured salmon sperm DNA but is highly sensitive to competition with synthetic dA-dT sequences.

The well characterized D1 protein from *Drosophila melanogaster* displays many similarities to BP1 and BP2. This is a nuclear protein with an apparent molecular weight on SDS gels of 64,000 (14) and which contains an unusually high proportion of basic and acidic amino acids. It is predominantly found *in vivo* associated with AT rich satellite DNA (14) and *in vitro* it displays highly selective binding to AT rich DNA (25).

The Dictyostelium genome has one of the highest known genomic contents of AT base pairs (77% A+T (26)). In synonymous codons A or T is almost always used and both intergenic and intervening sequences are extremely (>90%) A-T rich (23). Thus we would suggest that BP1 and BP2 are bound to intergenic regions of the Dictyostelium genome. Since a large proportion of the DNA in these regions is organized into relatively long tracts of poly(dA) and poly(dT) (23), the fact that DNA binding to BP1 and BP2 was much more sensitive to competition by poly(dA) poly(dT) than by poly(dA.dT) may be of some significance. In chromatin reconstitution experiments long (approximately 80 nucleotide) segments of poly(dA) poly(dT) are not folded into nucleosomes and even short (approximately 20 nucleotide) segments are distinctly disfavoured (27,28,29). Thus, while BP1 and BP2 might not have a role in controlling the expression of any particular gene they may play an important role in chromosomal organization. Further investigation of the proteins will require their purification and this is now in progress.

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