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The *cbpA* gene: Role of the 26,000-dalton carbohydrate-binding protein in intercellular cohesion of developing *Dictyostelium discoideum* cells

(cell aggregation/lectin)

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ABSTRACT **The loss of the lectin-like activity of a 26,000-dalton carbohydrate-binding protein (CBP-26) results in the loss of aggregation competence and cell–cell cohesiveness in developing cells of *Dictyostelium discoideum*. The lesion responsible for this phenotype behaves like a mutation in the structural gene for CBP-26, maps in linkage group II, and has been designated *cbpA1*. In aggregation-competent revertants, the degree of aggregation competence and cell–cell cohesiveness is directly related to the specific activity of CBP-26. Thus, CBP-26 appears to play an essential role in cell aggregation through the cell–cell cohesion process.**

In response to nutrient depletion, individual cells of the cellular slime mold *Dictyostelium discoideum* become cohesive and aggregate into a multicellular mass (1, 2). Because of its similarity to lectins involved in other cell adhesion systems (3, 4), a 26,000-dalton carbohydrate-binding protein (CBP-26) is thought to be involved in these processes. Biochemical evidence for its involvement is as follows: (*i*) CBP-26 is found in small amounts in noncohesive vegetative cells and in large amounts in cohesive cells (4–6); (*ii*) the time course of the 400-fold increase in the amount of CBP-26 is very similar to the time course for the acquisition of cellular cohesiveness (4–6); (*iii*) CBP-26 is located on the surface of cohesive cells (5–8); (*iv*) purified CBP-26 can agglutinate glutaraldehyde-fixed *D. discoideum* cells (8); and (*v*) species-specific cell cohesion correlates well with the ligand specificities of the carbohydrate-binding proteins (9). In addition, CBP-26 is absent in noncohesive mutants (6), and a mutant that produces nonfunctional CBP-26 fails to become cohesive (10). A caveat in these genetic studies is that cohesiveness is the result of a developmental program and the loss of any one step in the developmental sequence may prevent the acquisition of cohesiveness. To remove this caveat we performed a detailed analysis of the lesion responsible for the production of inactive CBP-26. The lesion behaves as a single-site mutation in the structural gene for CBP-26. In revertants of this lesion, the degree of aggregation and cohesiveness correlates well with the specific activity of CBP-26. Thus, CBP-26 activity appears to be involved in the acquisition of cohesiveness and may be directly involved in the intercellular cohesion process.

MATERIALS AND METHODS

Strains. Strain NC-4 was used as wild type (11). Strains HL501 (*cycA1 whi tsgD12 acrA1 nag man*) and HP10 (*tsgA bwn pds*) were obtained from W. F. Loomis (12). Strain HJR-1 (*cbpA1*) is described in ref. 10.

Genetic Analyses. Stable diploids were isolated by requiring complementation of *tsg* (temperature sensitive for growth) alleles (13–15). Diploid strains were identified by growth at 27°C and by spore size. Haploid segregants were selected by plating approximately 5×10^4 diploid amoebas together with *Klebsiella aerogenes* (a food source) on SM agar containing 2% (vol/vol) methanol or 500 μg of cycloheximide per ml. All diploids were heterozygous for one of these drug resistance markers. Where possible, the ploidy of the segregants was determined by measuring spore size.

Development of Cells. Cells were developed as described (16). Briefly, growing cells were harvested in cold distilled H_2O and separated from bacteria by repeated differential centrifugation. The final cell pellet was resuspended in 20 mM KCl/5 mM $MgCl_2$/10 mM potassium phosphate, pH 6.4, and the cell density was adjusted to 2×10^8 cells per ml. Cells were then placed on buffer-saturated Whatman no. 50 filter paper and incubated at 22°C. Synergy studies were done similarly except the two strains were mixed prior to placement on the filter paper (6, 10).

Cohesion Assay. After 15 hr of development on pads, amoebas were harvested in 17 mM potassium phosphate, pH 6.4/20 mM EDTA. The cell density was adjusted to 1×10^7 cells per ml. Equal volumes of the cell suspension and 17 mM potassium phosphate (pH 6.4) were mixed and the cells were shaken at 22°C (100 rpm, G-10 gyratory shaker). Samples were taken periodically and the number of single cells was determined in either a hemacytometer or a Coulter counter.

Determination of Specific Activity of CBP-26. The amount of CBP-26 was quantitated by radioimmunoassay with a standard curve constructed with purified CBP-26 (6). The activity of CBP-26 was measured in the sheep erythrocyte agglutination assay (5).

RESULTS

Mapping of *cbpA* Locus. Strain HJR-1 fails to aggregate into multicellular slugs (10). At the appropriate time during the developmental program, cells of this strain make wild-type amounts of CBP-26 and insert the protein into its membrane. However, CBP-26 fails to agglutinate sheep erythrocytes (10). This suggests that strain HJR-1 carries a lesion in the CBP-26 gene and that CBP-26 is involved in aggregation. We investigated the lesion(s) in this strain further by first isolating a derivative of it that was temperature sensitive for growth and then crossing this derivative to the multiply marked, aggregation-competent strain HL501. The diploid [HJR-1 (agg^-)/HL501 (agg^+)] exhibited the aggregation phenotype of the aggregation-competent parent; thus, the lesion(s) in HJR-1 is recessive to the wild-type allele(s).

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Abbreviation: CBP-26, 26,000-dalton carbohydrate-binding protein; NMG, *N'*-nitro-*N'*-methyl-*N*-nitrosoguanidine.

A parasexual cycle was used to map the lesion in HJR-1. The presence of CBP-26 activity was always associated with aggregation competence; that is, 29 of 29 aggregation-competent progeny displayed wild-type levels of CBP-26 activity and 21 of 21 aggregation-deficient progeny had no detectable CBP-26 activity. Furthermore, aggregation competence cosegregated with markers in linkage group II (*acrA, whi*) and segregated independently of markers in linkage groups I, IV, and VI (Table 1). Therefore, the lesion in HJR-1 behaves like a locus in linkage group II. Because this lesion results in the production of an altered CBP-26, it has been designated the *cbpA* locus and the mutant allele has been designated *cbpA1*. Because the resolution limit of this cross is 2 map units, this locus may contain either a single lesion or multiple lesions that are within 2% of each other.

Phenotype of Revertants of HJR-1. In order to probe further the relationship between CBP-26 and aggregation, we isolated 58 revertants of HJR-1 that had regained the ability to complete development. Such revertants arose at frequencies [approximately 10^{-8} spontaneous revertants per cell; 5×10^{-4} revertants per viable *N'*-nitro-*N'*-methyl-*N*-nitrosoguanidine (NMG)-mutagenized cell] characteristic of point mutations in *D. discoideum*. The 58 revertants fell into three discrete classes with respect to sorocarp formation, the end product of the developmental program. The 18 class I revertants were indistinguishable from the wild-type strain; that is, when grown on SM agar in association with *K. aerogenes*, amoebas began aggregating shortly after a visible plaque arose (plaque diameter 8–12 mm). When amoebal growth reached confluency, the plate contained many fruiting bodies (Fig. 1 *B* and *C*). [Mutant HJR-1 failed to form any fruiting bodies (Fig. 1*A*).] The 15 class 2 revertants began forming fruiting bodies slightly later (plaque diameter 17–23 mm) and at confluency displayed only 20–40% as many fruiting bodies as the wild-type strain (Fig. 1*D*). In addition, these fruiting bodies were significantly smaller than those of the wild type. The 25 class 3 revertants started forming fruiting bodies even later (plaque diameter 27–35 mm) and at confluency displayed only a few, small fruiting bodies (Fig. 1*E*).

A slightly different phenotype was observed when the revertants were analyzed under conditions that promoted synchronous development—i.e., when exponentially growing cells were removed from the nutrient source and placed on buffer-saturated pads at a density of $1\text{–}2 \times 10^7$ cells per cm^2 (14). The revertant strains exhibited essentially the same time course of aggregation, multicellular slug formation, and culmination as wild-type strains. However, the size and number of fruiting bodies depended on the class of revertant. Class I revertants formed the same number of and size sorocarps as wild type; class 2 revertants formed more but smaller sorocarps; and class 3 revertants formed even more and smaller sorocarps. Thus, the lesion(s) in the revertants appear to influence primarily the size of the fruiting bodies. The number of fruiting bodies varies with size because the same number of cells are participating in development.

Table 1. Analysis of HS15 (*tsg cbpA1 cycA^s acrA^s whi$^+$ nag$^+$ man$^+$*) × HL501 (*cbpA$^+$ cycA1 acrA1 tsgD12 whi nag man*)

Gene pair	No. of progeny found*	
	Parental	Nonparental
cbpA cycA	54	21
cbpA acrA	75	0
cbpA whi	54	0
cbpA nag	29	21
cbpA man	24	26

* Progeny were selected by cycloheximide resistance. Where possible, progeny were screened for ploidy and only haploid progeny were chosen for study. The markers were phenotypically scored as described (13, 14). These markers represent linkage groups I (*cycA*), II (*acrA, whi*), IV (*nag*), and VI (*man*).

Cohesiveness of Revertants of HJR-1. Because sorocarp size depends on the size of the initial aggregate (17) and because one of the factors involved in determining the size of the initial cell aggregate is the cohesiveness of the cells, we determined the cohesiveness of five revertants from each class by the standard assay (18, 19). Typical results are shown in Fig. 2. As previously reported (10), HJR-1 (*cbpA1*) cells were noncohesive and failed to form aggregates. On the other hand, wild-type NC-4 cells began forming aggregates immediately after suspension and within 15 min reached a plateau of 40% (±3%) single cells remaining. After 45 min of incubation, the average aggregate contained more than 100 cells. Class 1 revertants behaved indistinguishably from the wild-type strain. In the class 2 revertants, the rate of loss of single cells was reduced and fewer cells entered aggregates (i.e., there was a higher plateau level of approximately 50% single cells remaining). In addition, the aggregates at 45 min were smaller (20–50 cells per aggregate). Class 3 revertants were even less cohesive in that aggregates were formed more slowly, fewer cells entered aggregates, and the aggregates contained on the average only 5–10 cells. Thus, the cohesive properties of these cell populations correlated well with their developmental properties. Furthermore, because the size of aggregates formed paralleled developmental competence, these data suggest that this correlation is due to a variation in the cohesiveness of the cells themselves rather than to a variation in the number of cells proceeding through development to acquire wild-type cohesiveness. (A mixture of 10% NC-4 and 90% HJR-1 produced the same size aggregates as 100% NC-4.)

Specific Activity of CBP-26 in Revertants. The amount of CBP-26 lectin-like activity in each revertant was determined in the standard sheep erythrocyte agglutination assay (5, 6). In this assay, wild-type NC-4 cells displayed an agglutination titer of 190–200 units/mg of protein whereas the undiluted sample from HJR-1 (*cbpA1*) cells (2 mg of protein per ml) failed to agglutinate the sheep erythrocytes (Table 2). In the revertants of HJR-1, CBP-26 agglutination activity paralleled aggregation behavior (Table 2). That is, all class 1 revertants except HS31 displayed wild-type aggregation and wild-type levels of CBP-26 activity (175–225 units/mg of protein), class 2 revertants displayed intermediate levels of each (50–80 units/mg of protein), and class 3 revertants displayed little aggregation and little CBP-26 activity (8–20 units/mg of protein). The one exception, HS31, was isolated after NMG mutagenesis and displayed wild-type cohesion and development but had only 2–4 units of CBP-26 agglutination activity per mg of protein.

The increased level of CBP-26 activity in a revertant could be due to an increase in the number of CBP-26 molecules per cell or to an increase in the specific activity of the CBP-26 molecules. To distinguish between these possibilities we determined the amount of CBP-26 present by radioimmunoassay in five revertants from each class (Table 2). The amount of CBP-26 per cell was the same for the 15 revertant strains, mutant strain HJR-1, and wild-type strain NC-4. Thus, the increase in CBP-26 activity in the cell lysates represents an increase in the specific activity of the CBP-26 molecules (Table 2). Therefore, the aggregation behavior of the revertants is correlated to the specific activity of the CBP-26 molecules.

Mapping and Complementation Analyses of Revertants of HJR-1. We crossed a *tsg* (temperature sensitive for growth) derivative of each revertant to a *tsg* derivative of HJR-1. In each

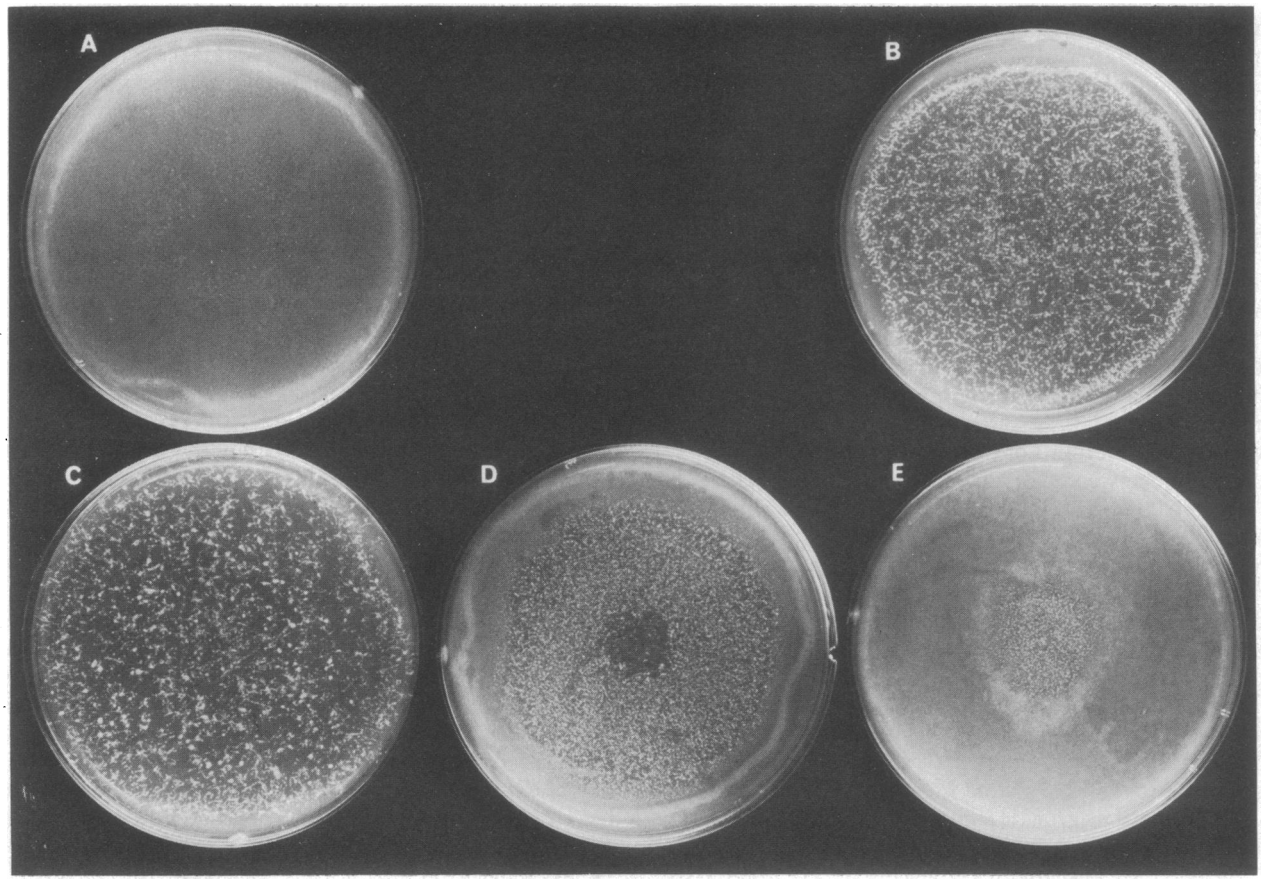

FIG. 1. Amoebas were inoculated into the center of an SM agar plate that had been spread with *K. aerogenes*. Plates were incubated at 22°C. Photographs were taken when the amoebas reached confluency. (*A*) HJR-1 (*cbpA1*); (*B*) NC-4 (wild type); (*C*) HS81 (class 1 revertant); (*D*) HS5 (class 2 revertant); (*E*) HS3 (class 3 revertant).

cross, one of the strains carried an *acrA*^{r} mutation (resistance to methanol). Diploids were successfully isolated in all crosses except those involving HS31. We were particularly interested in the behavior of this revertant because it displays the phenotype associated with one class of possible phenotypic suppressors (see *Discussion*). Unfortunately, we have been unable to obtain any stable diploids in 20 attempts at crossing HS31 to HJR-1 or HL501. Attempts to recover recombinants from

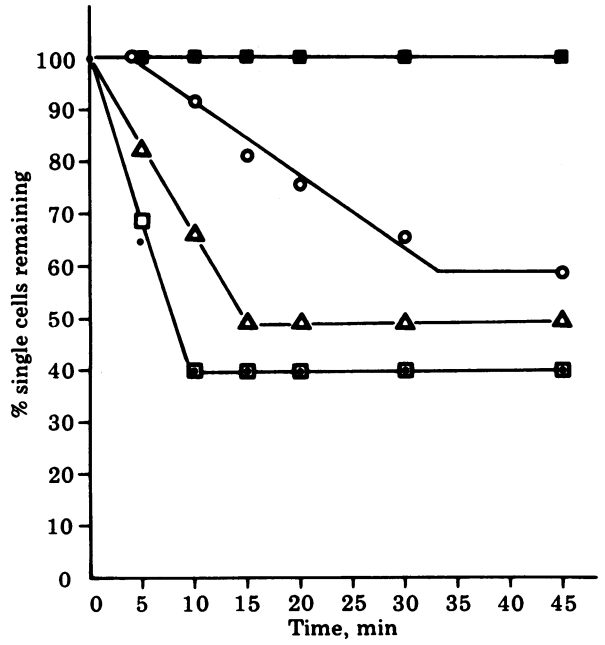

FIG. 2. Cohesiveness of 15-hr cells was measured as described (19). •, NC-4 (wild type); ■, HJR-1 (*cbpA1*); □, HS81 (class 1 revertant); △, HS5 (class 2 revertant); ○, HS3 (class 3 revertant). Each data point has an error of approximately ±3% single cells.

Table 2. CBP-26 specific activity*

Class	Strain	Activity/ mg protein	CBP-26, μg/mg protein	Activity/ μg CBP-26
Wild type	NC-4	212	5.4	39
Wild type	HL501	192	5.5	35
Mutant	HJR-1	<1	4.6	<0.22
Mutant	HS15	<1	5.3	<0.2
1	HS39	206	5.6	37
1	HS67	193	5.5	35
1	HS81	168	5.1	33
1	HS161	188	5.4	35
1	HS31	2–4	6.2	0.3–0.6
2	HS5	60	5.3	11
2	HS36	51	5.7	9
2	HS178	68	5.8	12
2	HS206	75	6.0	12.5
3	HS3	9.2	5.5	1.7
3	HS158	9	5.7	1.6
3	HS202	10.9	6.5	1.8
3	HS212	13	5.4	2.4

* The CBP-26 specific activity for wild-type, HJR-1 (*cbpA1*), and various revertants was calculated by dividing the CBP-26 lectin-like activity measured in the sheep erythrocyte agglutination assay (5, 6) by the amount of CBP-26 present as measured in a radioimmunoassay (6).

metastable diploids possibly formed in the cross HP10 × HJR-1 have also failed.

All diploids displayed the aggregation phenotype of the revertant. In this sense, the revertant alleles are dominant to the *cbpA1* allele. For each cross, at least 100 haploid, methanol-resistant progeny were selected and scored for their aggregation phenotype. In all crosses, the aggregation phenotype of the methanol-resistant parent cosegregated with methanol resistance (Table 3). Thus, the lesions in the revertants are in or linked to (within 2%) the *cbpA* locus.

For complementation analysis, temperature-sensitive, methanol-resistant derivatives of three revertants from class 2 and from class 3 were isolated. (Class 1 revertants were not analyzed for complementation because the class 1 revertant × HJR-1 diploids exhibited wild-type aggregation behavior.) Each derivative was then crossed to five revertants from both class 2 and class 3. All class 2 × class 3 diploids exhibited class 2 aggregation behavior (Table 3). All crosses among members of the same revertant class produced diploids that exhibited the same aggregation phenotype as the parents. Thus, because positive complementation is defined as wild-type aggregation behavior in the appropriate diploid, all class 2 and class 3 revertants fail to complement and, as such, are placed in the same complementation group. Furthermore, class 2 behavior is dominant to class 3 behavior.

Synergy Assays. One can measure the ability of cells to make the cell–cell contacts required for development by mixing two cell types and allowing them to develop together (20). If the proper contacts can be made, the ratio of the cell types in individual fruiting bodies will reflect the ratio of the cell types in the original mixture. For example, HJR-1 (*cbpA1*) is said to be able to synergize with HL501 (*cbpA*$^+$) because a 1:1 mixture of HJR-1 and HL501 cells results in a 1:1 ratio of HJR-1 and HL501 cells in individual fruiting bodies (Table 4). Similarly, all revertants except HS31 can synergize with both HJR-1 (*cbpA1*) and HL501 (*cbpA*$^+$) (data not shown). Cells of HJR-1 (*cbpA1*) do not interact properly with HS31 cells; essentially only HS31 spores are found in the fruiting bodies (Table 4). The few HJR-1 spores could arise either from "trapping" of HJR-1 cells in the HS31 multicellular aggregate or from weak interactions between HS31 and HJR-1 cells. Thus, it appears that revertant HS31 is altered in the component(s) required for synergy in addition to being altered in its CBP-26 activity.

Table 3. Analysis of lesions in the revertants

cbpA allele* of		Aggregation phenotype† of			No. of *acrA*^{r} progeny	
acrA^r parent	*acrA*^{s} parent	*acrA*^{r} parent	*acrA*^{s} parent	Diploid	agg$^+$‡	agg$^-$
A$^+$	A1	+	−	+	182	0
A1	A$^+$	−	+	+	0	161
A39	A1	1	−	1	0	202
A1	A39	−	1	1	311	0
A67	A1	1	−	1	0	155
A1	A81	−	1	1	138	0
A1	A161	−	1	1	219	0
A5	A1	2	−	2	0	142
A1	A5	−	2	2	192	0
A36	A1	2	−	2	0	239
A1	A178	−	2	2	185	0
A1	A206	−	2	2	231	0
A3	A1	3	−	3	0	235
A1	A3	−	3	3	220	0
A158	A1	3	−	3	0	215
A1	A202	−	3	3	106	0
A1	A212	−	3	3	170	0
A5	A36	2	2	2	81	0
A5	A3	2	3	2	76	0
A5	A158	2	3	2	68	0
A158	A5	3	2	2	56	0
A158	A36	3	2	2	93	0
A158	A178	3	2	2	88	0
A158	A206	3	2	2	61	0
A158	A3	3	3	3	79	0

* The *cbpA* allele number corresponds to the strain number—i.e., HJR-1 carries *cbpA1*, HS39 carries *cbpA39*, and wild-type carries *cbpA*$^+$. The *acrA*^{r} allele confers the ability to grow on SM agar containing 2% (vol/vol) methanol.

† The aggregation class is defined as shown in Fig. 1 and described in the text. Diploids were isolated as described (13–15).

‡ The aggregation phenotype (revertant class) of the agg$^+$ progeny was that of the methanol-resistant (*acrA*^{r}) parent strains.

DISCUSSION

The three classes of revertants of HJR-1 (*cbpA1*) can be distinguished by either the size of the developmental end product (the sorocarp) or the amount of CBP-26 activity present in cells developed for 12 hr. These two phenotypes are most likely related through the cell–cell cohesion process. That is, an alteration in CBP-26 activity is paralleled by an alteration in cell–cell cohesiveness. The degree of cohesiveness influences the size of the multicellular aggregate, which in turn determines sorocarp size. No sorocarps are formed in cultures of mutant strain HJR-1 (*cbpA1*) because the absence of CBP-26 activity correlates with an absence of cell–cell cohesiveness, thus preventing aggregate formation and, hence, subsequent development.

In a cascade-like developmental system, a correlation between the loss of a particular function and the loss of developmental competence indicates neither that the function is required for development nor that the function itself is altered because production of the function could be blocked due to an alteration in an earlier, required step in the cascade. However, cells of strain HJR-1 (*cbpA1*) and its revertants contain wild-type amounts of CBP-26-specific antigenic determinants. Hence, these strains must be able to proceed through all the developmental steps required for production of the CBP-26 molecules. The simplest explanation of the behavior of strain HJR-1 (*cbpA1*) and its revertants is that these strains carry lesions in the structural gene for CBP-26 whose activity is re-

Table 4. Synergy studies

Source of cells	No. found	
	Strain 1	Strain 2
HJR-1 + HL501		
Original mix	72	94
Slug 1	34	42
Slug 2	51	70
Sorus 1	43	56
Sorus 2	76	96
Total spores	56	64
HJR-1 + HS31		
Original mix	106	85
Slug 1	3	80
Slug 2	4	96
Sorus 1	1	81
Sorus 2	0	82
Total spores	3	90

Cells of HJR-1 (*cbpA1*) and HL501 (*cbpA*$^+$) or HJR-1 and HS39 (*cbpA39*) were mixed and allowed to develop on pads (6, 10). Individual slugs or sori were isolated and disrupted and their cells were assayed for phenotype as described (6, 10).

quired for cohesion and subsequent development. An alternate explanation is that the mutants carry lesions in a development process required for the activation of CBP-26 function. In this case, either CBP-26 activity could be essential for development or its correlation with developmental competence could be fortuitous. For example, the lesions in the mutants could be in an enzyme that activates both CBP-26 activity and a developmentally required function. Determination of the primary structure of the CBP-26 gene and of the mutant alleles will distinguish between these explanations of the correlation of CBP-26 activity and developmental competence.

One of the NMG-induced revertants, HS31, exhibits the class 1 revertant phenotype; i.e., it is indistinguishable from wild type with respect to aggregation, cell–cell cohesiveness, and sorocarp formation but has only 0.5 unit of CBP-26 agglutination activity per μg of CBP-26 (wild type has 35–40 units/μg). One possible explanation is that HS31 carries a phenotypic suppressor of the *cbpA1* lesion. Such suppression could result from an alteration in the ligand for the CBP-26 lectin-like activity such that it can bind to the mutant CBP-26. The inability of HS31 cells to interact properly with HJR-1 (*cbpA1*) cells in the synergy study (Table 4) suggests that both the lectin and ligand of the postulated CBP-26 cohesion system are altered in HS31. Furthermore, preliminary experiments suggest that the putative ligand in HS31 either is altered in such a way as to act no longer as a ligand for wild-type CBP-26 or is produced in much lower amounts. This raises the intriguing possibility that HS31 has "evolved" a private lectin–ligand recognition system. This could explain the heretofore difficult concept of the evolution of lock-and-key interactions. In other words, when a mutation in the lock is suppressed by a complementary mutation in the key, two consequences follow: (*i*) normal recognition is restored because the new key recognizes the new lock and (*ii*) the key fits no other locks, leading to private recognitive specificity. Obviously a series of such suppressor mutations could lead to the multiplicity of private cell–cell interactions. Indeed, such pairs of complementary mutations are thought to generate novel mating specificities in *Physarum polycephalum* (unpublished observations; R. Anderson and C. Holt, personal communication). An alternate explanation of the phenotype of HS31 is that the revertant lesion allows HS31 to bypass the apparent requirement of CBP-26 activity for intercellular cohesion and subsequent development. For example, if the interaction of CBP-26 with its receptor acted as a signal for further development, then in HS31 the receptor could be altered such that it always "signals" that it has interacted with CBP-26. Clearly, further biochemical and genetic analyses of this particularly interesting mutant could lead to a better definition of the role of CBP-26 in development and, possibly, to identification of the ligand for CBP-26.

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