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The Regulatory C Proteins from Different Restriction-Modification Systems Can Cross-Complement

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The *Bam*HI restriction-modification system contains a third gene, *bamHIC*, which positively regulates *bamHIR*. Similar small genes from other systems were tested in vivo for their ability to cross-complement. C.*Bam*HI protein was identified, purified, and used to raise polyclonal antibodies. Attempts to detect other C proteins in cell extracts by cross-reactivity with C.*Bam*HI antibodies proved unsuccessful.

The characterization of type II restriction-modification (RM) systems have shown some to contain components in addition to the requisite restriction endonuclease (*R*) and modification methylase (*M*) genes (20). One such system is *Bam*HI from *Bacillus amyloliquefaciens* H, recognizing the sequence GGATCC (9, 19). The system was cloned in this laboratory (2), and nucleotide sequence analysis showed the endonuclease (*bamHIR*) and methylase (*bamHIM*) genes to be divergently oriented (3). Between them lay a 306-bp open reading frame, which was designated *bamHIC* (for controlling element) since it was found to stimulate *bamHIR* and repress *bamHIM* expression in clones of the system (6, 8). A small gene with positive regulatory function was also reported in the *Pvu*II RM system and designated *pvuIIC* (14). The derived amino acid sequence of the product, C.*Pvu*II, was homologous to that of C.*Bam*HI; furthermore, similar genes were found in two other RM systems, *Eco*RV and *Sma*I (14). These have been designated *C* genes by analogy, but no function for these genes has been identified (1, 5). All are found in RM systems where the *R* and *M* genes are either divergently or convergently oriented; *C* is always upstream of and in close proximity to *R* (14).

In this study we tested the ability of heterologous *C* genes to substitute in the cloned *Bam*HI and *Pvu*II RM systems. We identified the C.*Bam*HI protein product, and after its overexpression and purification, assessed whether C.*Bam*HI-specific antibodies raised against the purified protein can detect C proteins in extracts of other RM systems.

Interspecific *C* gene substitution in the *Bam*HI and *Pvu*II RM systems. *C* gene cross-complementation was first tested with the *Bam*HI system cloned in *Escherichia coli. E. coli* ADK21 cells which carry, on a λ lysogen, an extra copy of *bamHIM* necessary for *Bam*HI RM maintenance (3, 7) were transformed with pIHBamRMdC, a pACYC-derived vector containing the *Bam*HI RM system with *bamHIC* disrupted. In a second step, the cells were transformed with a compatible pUC vector containing a *C* gene subcloned, by standard cloning techniques (10), from the *Bam*HI, *Pvu*II, *Eco*RV, or *Sma*I system (Table 1). Cells were grown, and extracts were prepared and assayed for endonuclease (R.*Bam*HI) activity as previously described (2, 15). The results are shown at the top of Table 2.

When *bamHIC* was disrupted, endonuclease activity in the extract dropped at least 200-fold, below the limit of detection. Providing *bamHIC* on a second plasmid partially restored R.*Bam*HI activity, as had previously been shown (6). Substitution of *smaIC* for *bamHIC* also resulted in partial restoration of R.*Bam*HI activity, demonstrating that complementation could in fact occur with a heterologous *C* gene. An intact *smaIC* was necessary; when a plasmid carrying *smaIC* which had been disrupted by insertion of a *Bgl*II linker at the *Sca*I site was substituted, no R.*Bam*HI activity was detected. The *pvuIIC* gene was also able to complement the *bamHIC* mutation, but not as well as either *bamHIC* or *smaIC*. When *ecoRVC* was used, no R.*Bam*HI activity could be detected.

The fact that *smaIC* as well as *pvuIIC* and *bamHIC* can function in the *Bam*HI system strongly suggests that it also serves as a positive regulator of endonuclease function in the

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristic(s)	Source or reference[a]
pUC19	Cloning vector	21
pACYC184	Cloning vector	4
pBamRM5.0	pACYC184 containing *bamHIM bamHIR bamHIC*	2
pIHBamRMdC	pACYC184 derivative containing *bamHIM bamHIR* with *bamHIC* disrupted	I. Hall; (6)
pUCBamC	pUC19 containing *bamHIC*	(6)
pBamC	pACYC184 containing *bamHIC*	6
pUCSmaIC	pUC19 containing *smaIC*	(5)
pUCSmaIdC	pUC19 containing *smaIC* disrupted by *Bgl*II linker	(5)
pKL141RM103-11	pUC19 containing *smaIM smaIR smaIC*	K. Lunnen; 5
pUCPvuIIC	pUC19 containing *pvuIIC*	(14)
pPvuRM3.4	pBR322 containing *pvuIIM pvuIIR pvuIIC*	14
pPvuIIRM3.4-Cla35	pBR322 containing *pvuIIM pvuIIR* with *pvuIIC* disrupted	14
pPvuCYC2.6	pACYC184 containing *pvuIIM pvuIIC*	14
pUCEcoRVC	pUC19 containing *ecoRVC*	(17)
pEcoRVRM8.7-2	pBR322 containing *ecoRVM ecoRVR ecoRVC*	17
pBamM2.2	pUC19 containing *bamHIM bamHIC*	2
pBamRMdC	pUC19 containing *bamHIM bamHIR* with *bamHIC* disrupted	6
pPN862	pUC19 containing kanamycin cassette	(18)
pBamCX-19	pPN862 derivative containing *bamHIC*	(6)
pBamCS-19	pPN862 derivative containing *bamHIC*	(6)

[a] Reference numbers in parentheses indicate the reference for the DNA inserted into the cloning vector listed to generate the plasmid designated.

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TABLE 2. Restoration of *Bam*HI or *Pvu*II endonuclease activity by *C* gene complementation[a]

Endonuclease	Primary plasmid (and genotype)	Secondary plasmid (and genotype)	Endonuclease activity (U/g [wet wt])
*Bam*HI	pBamRM5.0 ($R^+M^+C^+$)		6.0×10^3
	pIHBamRMdC ($R^+M^+C^-$)		$<3 \times 10^1$
	pIHBamRMdC ($R^+M^+C^-$)	pUCBamC (*bamHIC*$^+$)	1.7×10^3
	pIHBamRMdC ($R^+M^+C^-$)	pUCSmaIC (*smaIC*$^+$)	4.3×10^3
	pIHBamRMdC ($R^+M^+C^-$)	pUCSmaIdC (*smaIC*$^-$)	$<3 \times 10^1$
	pIHBamRMdC ($R^+M^+C^-$)	pUCPvuIIC (*pvuIIC*$^+$)	2.0×10^2
	pIHBamRMdC ($R^+M^+C^-$)	pUCEcoRVC (*ecoRVC*$^+$)	$<3 \times 10^1$
*Pvu*II	pPvuIIRM3.4 ($R^+M^+C^+$)		1.3×10^4
	pPvuIIRM3.4-Cla35 ($R^+M^+C^-$)		$<3.0 \times 10^1$
	pPvuIIRM3.4-Cla35 ($R^+M^+C^-$)	pPvuCYC2.6 (*pvuIIM*$^+C^+$)	4.8×10^3
	pPvuIIRM3.4-Cla35 ($R^+M^+C^-$)	pBamC (*bamHIC*$^+$)	6.4×10^3

[a] Cultures (50 ml) of ADK21 cells (*Bam*HI) or ER2267 cells (*Pvu*II) containing these plasmids were grown to mid-log phase and harvested. Endonuclease activity was assayed from crude extracts as described in reference 15. All experiments were done by growing and assaying three independent colonies; the means of the three assays are reported.

*Sma*I RM system. In contrast, the fact that the *ecoRVC* gene was unable to complement raises the possibility that it serves a different function in the *Eco*RV system or perhaps that it is an inactive "evolutionary artifact."

*Pvu*II was the next system tested, since it was known that *pvuIIC* was able to act in *trans* and the necessary *Pvu*II constructs were available (14). The experimental design was essentially the same as that for the *Bam*HI complementation experiments, except that *E. coli* ER2267 (Mcr$^-$ Mrr$^-$ Δ*lacZ*; New England Biolabs collection) was used as the host. The results are shown at the bottom of Table 2.

As in the *Bam*HI system, disruption of *pvuIIC* led to a loss of detectable endonuclease activity. When *pvuIIC*, along with *pvuIIM*, was provided on a second plasmid, R.*Pvu*II activity was partially restored. Substitution of *bamHIC* on a second plasmid was also able to restore R.*Pvu*II activity; *bamHIC* complemented the *pvuIIC* mutation with high efficiency. It is interesting to note that in the *Pvu*II system *bamHIC* complemented the *pvuIIC* mutation very well, whereas in the *Bam*HI system the *pvuIIC* gene complemented the *bamHIC* mutation poorly. The reason for this "nonreciprocity" is unknown.

C.*Bam*HI identification by in vitro transcription-translation. An in vitro *E. coli* transcription-translation system (Amersham) was used to identify C.*Bam*HI protein. CsCl-purified plasmids carrying components of the *Bam*HI system, with the *C* gene intact or disrupted (Table 1), were used as substrates. Two plasmids, pBamCX-19 and pBamCS-19, carrying *bamHIC* in opposite orientations, were constructed for this experiment. They were generated by inserting a *bamHIC* fragment (6) into the ampicillin gene of a pUC19 derivative carrying a kanamycin cassette (18) at the *Bam*HI site, by standard subcloning techniques (10). Plasmids were incubated under recommended conditions in a reaction buffer containing [^{35}S]methionine (>1,000 Ci/mmol; Amersham). An autoradiogram of the translation products is given in Fig. 1. A band corresponding in size to that expected for the *bamHIC* gene product (lanes F and G) was observed when pBamCS-19 and pBamCX-19 were used as substrates. Since the intensity of the band was the same from both constructs, the level of expression seems to be independent of the gene's orientation within the vector. The same band was observed when pBamM2.2, a plasmid containing both *bamHIC* and *bamHIM*, was used as the substrate (lane H). The identity of C.*Bam*HI protein was further confirmed by analyzing the products of plasmid pBamRMdC (lane E), where *bamHIC* was disrupted and the appropriate band disappeared.

The C.*Bam*HI protein seen in the autoradiogram was approximately 10 kDa; this matches well with the 9.4-kDa protein predicted if the translational start of C.*Bam*HI were the first ATG within the open reading frame (bp 1719 [3]).

Cross-reactivity of C.*Bam*HI-specific antibodies. Since the C proteins are similar in amino acid sequence, it seemed possible that C.*Bam*HI-specific antibodies could be used to identify C proteins in extracts of other RM systems. To produce C.*Bam*HI in large quantities for purification, *bamHIC* was cloned into the pMal-c vector and C.*Bam*HI was produced as a fusion protein with maltose-binding protein (New England Biolabs). After affinity purification and cleavage with factor Xa

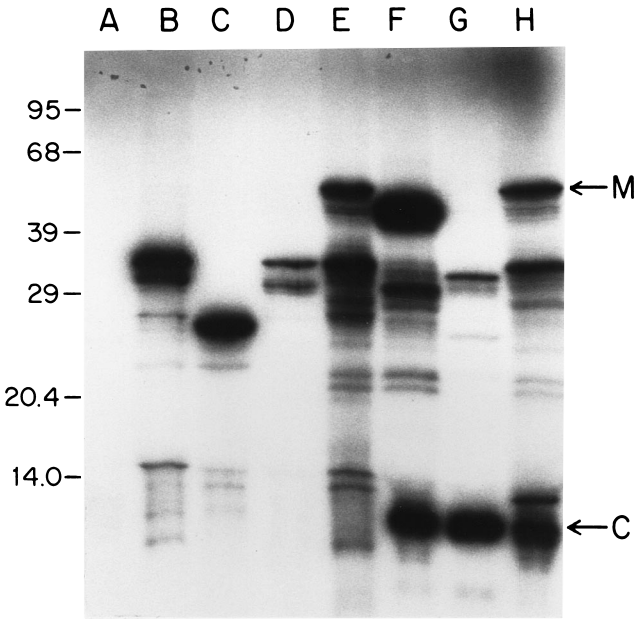

FIG. 1. Identification of C.*Bam*HI by a coupled transcription-translation assay in vitro. The synthesized proteins were labeled with [^{35}S]methionine, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17 to 27% gradient gel; Integrated Separation Systems), and detected by autoradiography with XAR5 film (Kodak). Migration of the molecular mass marker proteins (Integrated Separation Systems) is indicated in kilodaltons to the left of the figure. The CsCl-purified plasmids used in each reaction sample were as follows: lane A, no DNA; lane B, pUC19; lane C, pACYC184; lane D, pPN862; lane E, pBamRMdC; lane F, pBamCX-19; lane G, pBamCS-19; lane H, pBamM2.2. M.*Bam*HI (M) and C.*Bam*HI (C) proteins are indicated by arrows.

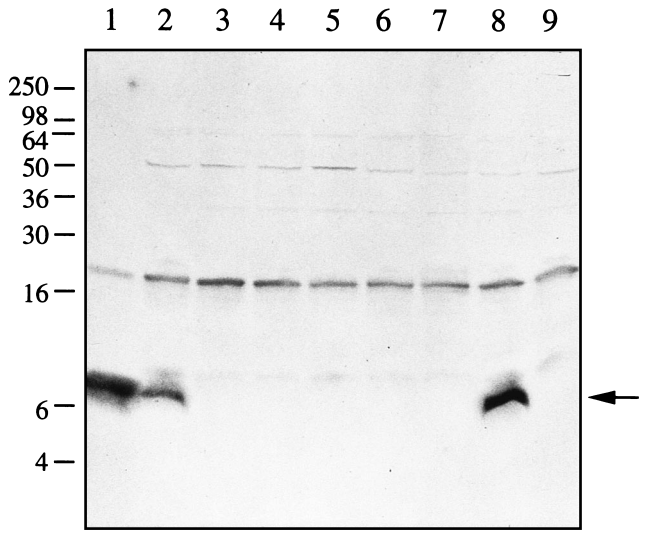

FIG. 2. Western blot of proteins from cloned RM systems using antiserum raised against C.*Bam*HI. Extracts of *E. coli* containing the plasmid(s) listed were prepared as described in reference 15. Samples containing 4 μg of total cellular protein were fractionated on a 16% Tricine gel (Novex). Proteins were electrophoretically transferred to a nitrocellulose membrane as described in reference 16. Incubations were performed with the mouse antibodies diluted 1:200 followed by biotinylated antimouse immunoglobulin G antibodies, as recommended by the manufacturer (Vector). Bound antibodies were detected with an avidin-biotin-peroxidase system (Vectastain ABC kit, Vector). The blot was developed with 4-chloro-1-naphthol (Sigma). Lanes: 1, purified C.*Bam*HI (0.5 ng); 2, pPvuIIRM3.4-Cla35 + pBamC; 3, pPvuIIRM3.4-Cla35 + pPvuCYC2.6; 4, pPvuIIRM3.4-Cla35; 5, pPvuIIRM3.4; 6, pEcoRVRM8.7-2; 7, pKL141RM103-11; 8, pBamRM5.0; 9, *E. coli* ER2267 extract. The arrow to the right indicates 9.4-kDa C.*Bam*HI protein. The 22-kDa band visible in lanes 2 through 9 is the *E. coli* biotin carboxyl carrier protein (12). Numbers to the left refer to molecular mass standards (in kilodaltons).

(as recommended by the supplier), C.*Bam*HI was purified to near homogeneity (11). Two samples (100 μg each) of purified C.*Bam*HI were injected intraperitoneally per mouse, and the pooled sera (diluted 1:200) were used to probe Western blots (immunoblots) of the cloned RM systems. The plasmids carrying the cloned RM systems are listed in Table 1. The results are shown in Fig. 2. A good signal from C.*Bam*HI was seen in extracts of the cloned *Bam*HI system (lane 8). Also, when plasmid-borne *bamHIC* was used to complement the *Pvu*II system with *pvuIIC* disrupted, a prominent band was seen (lane 2). However, no cross-reactivity was seen against C proteins in extracts of the *Pvu*II, *Sma*I, or *Eco*RV systems (lanes 3 through 7) probed with C.*Bam*HI antiserum at the same dilution. It is possible that the other C proteins are not expressed as well as, or as stably as, C.*Bam*HI in *E. coli*. However, even if the proteins are present in substantial amounts, the cross-reactivity of the heterospecific C proteins may be significantly lower than reactivity with C.*Bam*HI. Therefore, it may be necessary to use higher concentrations of antiserum to detect binding to the other C proteins. This will have to await further purification and concentration of the C.*Bam*HI-specific antibodies, since at higher concentrations of the antiserum as prepared, binding to nonspecific *E. coli* proteins obscured the results (data not shown).

Where do the C proteins act? It is likely that the C proteins specifically bind DNA at a site (or sites) within their respective RM systems, although no such binding site has yet been identified. The cross-complementation experiments suggest that the *Bam*HI, *Sma*I, and *Pvu*II (but not *Eco*RV) RM systems have homologous sites of action. The three systems were searched for short regions (approximately 10 to 20 bp in length) with common nucleotide sequences as candidate binding sites; special attention was given to the region just upstream of the *R* genes, where experiments in the *Pvu*II system suggest that its C protein is acting (13, 14). However, no common sequences could be identified. Experiments are now being done with the purified C.*Bam*HI using DNA from the *Bam*HI, *Pvu*II, and *Sma*I RM systems to identify the site (or sites) of action.

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