Balbani ring induction in phosphate metabolism

(cloned DNA/repetitive sequences/secretory proteins/amino acid composition/phosphoserine)

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ABSTRACT Balbani rings (BR), giant puffs in *Chironomus* larval salivary glands, code for giant secretory proteins. As shown earlier, the normally dominant BR2 is turned off with its putative translation product during exposure of larvae to compounds that diminish the stores of P_i . A BR6 develops from a compact chromosome band, and a new giant protein appears in the secretion as the major component. We have determined the sequence of cloned DNA fragments representative for large parts of BR1 and BR2 (normally active) and the inducible BR6. There is an excess of positive charges and high contents of serine/threonine in the coded amino acid composition for the BR1 and BR2 sequences. The coded amino acid sequence for the BR6 clone shares homologies with the others but has an excess of negative charges and lacks serine/threonine. This suggested that the P_i effects observed earlier could be related to differences in phosphorylation between the normal proteins and the BR6 product. This could be confirmed by measurements of phosphorylation, which occurs in the normal giant proteins mainly at seryl residues. P export with giant secretory protein is normally quantitatively important. Thus, BR6 activation should decrease P loss when P_i pools are lowered because of inducer action.

Balbani rings (BR) in polytene chromosomes of salivary glands from *Chironomus* larvae are genetic determinants of secretory proteins (1, 2). The large BRs (BR1 and BR2) produce giant messengers (3, 4) and code for giant secretory polypeptides, sp-Ia and sp-Ib (5, 6). If larvae are raised in solutions with monosaccharides or some other compounds, including glycerol, a BR6 develops from a condensed band and BR2 regresses (7, 8). Simultaneously a new giant protein appears (sp-Ic), immunologically different from sp-Ia and sp-Ib, and the labeling of sp-Ib decreases (8). A new giant RNA species also shows up, similar in size to the RNA from BR1 and BR2 (75S RNA) (9). The sp-Ic may account for 25% to more than 50% of the total gland incorporation of methionine (ref. 8 and this paper). P_i decreases in the hemolymph in response to inducers, and addition of P_i to the inducing solution abolishes the action. The induction also can be reversed with P_i (9).

In order to relate the P_i effects to the properties of the genes, we have cloned short fragments of BR1, BR2, and BR6 representing considerable parts of the genes (15–20 kb). The base sequences of these fragments suggested that phosphorylation might occur in the normal polypeptides, sp-Ia and sp-Ib, but not in sp-Ic. Direct analyses confirmed that suggestion. Because the P content in sp-Ia and sp-Ib is high and secretory protein export is quantitatively important, the switch to sp-Ic (BR6) implies that P is saved when, because of inducers, the P_i pool is low.

MATERIALS AND METHODS

Animals. Mid-fourth-larval instar *Chironomus pallidivittatus* animals were used, raised as described for *Chironomus*

tentans (10). For squashes, animals from single egg-string cultures were used; for other experiments, animals from mass cultures were used. Glycerol inductions were performed for 6 days with 50 mM glycerol/0.04% NaCl and otherwise under conditions as described (7).

Isolation of Nucleic Acids. DNA of *C. pallidivittatus* larvae (11), total gland RNA, and 75S RNA (5) were prepared. Plasmid DNA was isolated from cleared lysate by ultracentrifugation on cesium chloride/ethidium bromide gradients (12).

Labeling Procedures. Up to 1 μ g of 75S RNA was incubated for 10 min at 95°C in 50 mM Tris-HCl (pH 9.5) and chilled on ice. [γ - 32 P]ATP (Amersham; 50 μ Ci) was added, and allowed to react for 30 min at 37°C in 25 mM Tris, pH 9.5/2 μ M ATP/10 mM $MgCl_2$ /5 mM dithiothreitol containing 5 units of T4 polynucleotide kinase (Boehringer Mannheim). Free ATP was removed by chromatography on Sephadex G-100. Specific activity was $1\text{--}5 \times 10^7$ cpm/ μ g of 75S RNA.

Plasmid DNA was nick-translated with two deoxynucleoside triphosphates having specific activities of $1\text{--}5 \times 10^7$ cpm/ μ g (3 H) and $0.5\text{--}1 \times 10^8$ cpm/ μ g (32 P) (13).

Construction of Hybrid Plasmids. Total larval DNA was cut with *EcoRI* restriction endonuclease and separated on linear 5–24% NaCl gradient (11). Fractions enriched for BR sequences were identified by agarose gel electrophoresis and Southern blotting (75S [32 P]RNA as probe). DNA of >40 kilobases (kb) from positive fractions was digested with *Rsa* I, followed by blunt-end cloning in the dephosphorylated *HincII* site of pUC8 (14). Recombinants were screened with 75S [32 P]RNA as probe (15), and one clone, pCp 90, was further analyzed. It is a BR1-specific DNA fragment. For cloning BR6 DNA, 100 μ g of sheared DNA (2–25 kb) was fractionated on a linear NaCl gradient (see above), and DNA of <10 kb was collected and digested with *Bam*HI, *Hind*III, and *Eco*RI. After a second NaCl gradient fractionation, fractions were analyzed by agarose gel electrophoresis and Southern blots with 75S [32 P]RNA as probe. A 3- to 5-kb fraction gave strong hybridization. This DNA was digested with an excess of *Hin*FI to generate sticky ends, which were filled up with DNA polymerase I (Klenow fragment) for blunt-end cloning in the *HincII* site of pUC8. Eight clones were selected by colony hybridization with 75S [32 P]RNA (15), and one, pCp 16, was identified as BR6-specific. After the sequence of the whole insert of 902 base pairs (bp) had been determined, a fragment of 145 bp (132-bp insert and a 13-bp vector) was subcloned in pUC8 (clone pCp16-7) for blotting experiments. It extends from the *Hind*III site in the vector to the first *Sau*3AI site in the insert (Fig. 3) and includes part of a BR6 repeat unit. In all cases, recombinant plasmids were propagated in *Escherichia coli* HB101. L2/B2 German recombinant DNA guidelines were followed.

Sequence Determination. The sequence of cloned DNA was determined with both 3'- and 5'-end-labeled fragments after digestion with either *Hind*III or *Bam*HI, the restriction sites of which are in the polylinker of pUC8 (14). The 5' end-labeling is described elsewhere (16), whereas the 3'-end-la-

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Abbreviations: BR, Balbani ring; kb, kilobase (pairs); bp, base pairs.

beling was done with 2–5 pmol of the DNA fragment in 100 mM KPO₄, pH 7.0/100 mM KCl/7 mM MgCl₂ containing 2 μ M of one α -³²P-labeled nucleoside triphosphate and 5 units of Klenow enzyme. After 15 min at 20°C, nonreacted nucleotides were removed by chromatography on Sephadex G-100, the DNA was precipitated with ethanol for a second cut with another restriction enzyme and then was run on a 5% preparative polyacrylamide gel. DNA fragments were electroeluted from gel pieces into dialysis bags, precipitated with ethanol, and cleaned in a Sephadex G-100 column or on DE-52 resin by elution with 0.5 M NaCl. After precipitation and washing, they were used for sequence determinations (16). In all cases, the sequence of both strands of the inserts were determined in 75 cm $\times$ 0.2 mm gels (17).

In Situ Hybridization. Squashes were denatured with 0.07 M NaOH for 3 min before hybridization with nick-translated cloned DNA labeled with tritium (about 10⁷ cpm/ μ g) or with biotinylated UTP (18). For tritiated probes, hybridization was done at 60°C overnight in 2 $\times$ NaCl/Cit (1 $\times$ NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate, pH 7), whereas biotinylated probes were hybridized overnight at 58°C in 0.6 M NaCl/0.05 M sodium phosphate buffer, pH 7.2/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.2% bovine serum albumin/5 mM MgCl₂/0.2 mg of sonicated *E. coli* DNA per ml. Hybridized biotin was treated with goat anti-biotin IgG (Enzo Biochemicals, New York), and bound primary antibody with rhodamin-labeled rabbit anti-goat IgG (Miles).

Analysis of Phosphorylated Protein. One gland from each of six animals was used for incubation in 7 μ Ci of [γ -³²P]ATP (5000 Ci/mmol) in 33 μ l of modified Cannon solution (19). Sister glands were incubated similarly but with 7 μ Ci of [³⁵S]methionine (800 Ci/mmol) (Amersham). After 60 min, the glands were fixed and processed for electrophoresis in composite NaDodSO₄ gels (10) or 2% agarose gels with the identical buffer system.

For identification of protein-bound ³²P, 30 glands were labeled with ³²PO₄ (1 mCi/100 μ l of Cannon solution) for 30 min. Glands were processed and chromatographed on a Sepharose 6B column (5). An aliquot of 300 μ l of the pooled peak fractions (3 ml) containing sp-I was run on Bio-Gel P2 column to change to 0.05 M Tris-HCl buffer, pH 7.2. The protein, present in V₀, was divided into three equal fractions, which were analyzed by electrophoresis directly or after pronase and alkaline phosphatase digestions (20). Two other aliquots, each 500 μ l, were used for identifying phosphorylated amino acids. Five unlabeled glands dissolved in 50 μ l of buffer B (0.1 M Tris PO₄, pH 7.2/0.1 M dithiothreitol/0.02 M EDTA/3% NaDodSO₄) were added to each aliquot as carrier and 100% CCl₃COOH were added to 10%. The samples were allowed to precipitate overnight at 0°C and then were centrifuged (Sorvall rotor SS34 at 10,000 rpm for 30 min), and the supernatant was removed. To each tube, 300 μ l 6 M HCl was added for hydrolysis for 2 hr at 110°C, after which the HCl was removed over KOH. The hydrolysate was dissolved in HAc/HCOOH/H₂O, 78:20:902 (vol/vol), containing phosphoserine and phosphothreonine at 1 mg/ml. Aliquots (1 μ l) were placed for TLC (MN Cel 300).

Quantitative Determination of Alkali-Labile Phosphate. Secretion was isolated manually from fixed glands and was controlled by microscopy to be free from cellular material. For each analysis, 5–10 glands were used. The secretion was dissolved in 10–20 μ l of buffer B per gland (10). CCl₃ COOH then was added to 16%. After 30 min at 4°C, the precipitate was assembled by a 5-min centrifugation in an Eppendorf centrifuge, washed with 16% CCl₃COOH and extracted with CHCl₃/CH₃OH, 1:1 (vol/vol), CHCl₃/CH₃OH/concentrated HCl 2:1:0.01 (vol/vol), and diethylether (21). Treatment with CCl₃COOH at 90°C was avoided because it led to considerable protein losses and because the material was free of cellular debris and presumably also nucleic acid *P*. The pre-

cipitated secretory proteins were dissolved in 1 M NaOH, and two aliquots were taken for protein determination with Coomassie blue (22), with transferrin as standard. Two other aliquots were boiled for 15 min for P_i determination (23).

For *P* determination on sp-I protein, the secretion was isolated from 150 fixed glands, and proteins were dissolved and applied to 2 $\times$ 85 cm Sepharose 6B-CL column. The protein in the void volume, which contains only sp-I (5), was precipitated in 10% CCl₃COOH at 0°C overnight, collected by centrifugation, washed twice with 10% CCl₃COOH, once with 70% acetone, and once with 100% acetone. After being dried *in vacuo*, precipitates were dissolved in 1 M NaOH for 15 min at 65°C, after which aliquots were taken for protein and *P* analysis as described above; the aliquots for *P* analysis were taken after heating at 100°C for 15 min.

The validity of the Coomassie blue technique for secretory protein was controlled by relating the *A* values with corresponding measurements on total secretory proteins prepared as for *P* determinations, on which the nonlipid dry weight was determined. When data were corrected for the presence of carbohydrate [shown to be 17.4% for sp-I (24)] and for a *P* content of 5% (wt/wt) (present data for sp-I), the secretory protein gave a color intensity that was 86% of that of the transferrin on an equal weight basis. The primary data were adjusted to take this factor into account.

RESULTS

The BR1 Insert. The clone pCp90 contains a 231-bp insert. It hybridized to BR1 in *C. pallidivittatus* with slight cross-hybridization to BR2 (Fig. 1). The DNA represented by the insert consists of repeats tandemly arranged in the genome. This is apparent from Southern blots with genomic DNA digested to completion or partially with *Hinf*I restriction endonuclease, which cuts once per repeat (Fig. 2, lanes 2 and 3). This also shows that the repeat unit is similar in size to the insert. When similar blots were made with genomic DNA digested with a mixture of *Alu* I and *Sau*3AI, which have no recognition sites in the repeat, a nondigested hybridizing band of 15 kb was seen (Fig. 2, lane 1). Titration of the number of repeats as described (27) and as shown in Fig. 2 for BR6 suggested that the repeat is represented 69 times (average of 72 and 66). This value, accounting for 16 kb, agrees well with the size of the nondigested band, about 15 kb. Partial digests showed ladders (Fig. 2, lane 2) with up to 11-mers as a minimum value for a tandem arrangement. By assuming a size of 37 kb for BR1 as for BR2 in *C. tentans* (28), the cloned repeat represents about 40% of the gene. Only one open reading frame was present with a constant and a subrepeated part as in other BRs (11, 27, 29–32).

The restriction sites used for cloning are in the constant part (Fig. 3). The two cuts are probably in phase with the tandemly arranged repeat structure because the end of the insert could be joined to its beginning to yield a constant part homologous with other BR repeats. The subrepeated part contains seven six-codon repeats (Fig. 3). The six-codon repeat, in turn, consists of two similar three-codon repeats and is reminiscent of the pCt63 subrepeat from BR2 of *C. tentans* (29). The coded amino acid composition shown in Table 1 with high content of lysine, arginine, serine, and proline is similar to the composition for sp-Ia and -Ib in *C. tentans* (24).

The BR2 Insert. The sequence of pCp41 (11), which was revised, is given in Fig. 3. It represents a repeat unit of 195 bp. It is member of a family of repeats among which the constant parts show close to 100% homology and the subrepeated parts show only about 80% homology (11). To minimize a possible complication in the titration experiments due to this difference, we used as probe pCp31 from this family. This contains all of the constant part but only about half of the subrepeated part. It was represented 89 times (average of 71

FIG. 1. *In situ* hybridization of nick-translated plasmid pCp90 (with tritiated dATP and dCTP) and pCp16 (with biotinylated UTP) with *C. pallivittatus* salivary gland chromosomes. (Left) An autoradiography with pCp90 after an 8-wk exposure. (Right) Simultaneous phase and fluorescence microphotograph with pCp16. Roman numerals III and IV refer to chromosomes III and IV, respectively.

and 106) per haploid genome. Using restriction endonucleases without sites in the repeat (*Alu* I and *Rsa* I), we could not isolate a single block as for pCp90 (Fig. 2, lane 5), although a ladder suggesting a tandem arrangement has been observed in partial digests of genomic DNA (11). The repeat represents 17 kb or about half of the gene (assuming one gene per genome). The constant part of the pCp41 repeat shows 68% homology with the pCp90 constant part, although the respective subrepeated parts only show short

FIG. 2. Genomic structure of BR1, BR2, and BR6 genes of *C. pallivittatus*. Total larval DNA was cleaved partially or to completion with several restriction endonucleases, run on 1.4% (BR1/pCp90 and BR2/pCp41) and 1.5% (BR6/pCp16) agarose gels, blotted onto nitrocellulose filters (25) and hybridized with ³²P-labeled plasmid DNA containing BR gene fragments (26): for BR1, pCp90; for BR2, pCp31 (11); and for BR6, pCp16-7. Lanes: 3, 4, and 8, complete digests of total DNA with *Hinf*I; 2 and 7, partial digests (0.5×) obtained by shorter digestion times [the partial digestion for the BR2 gene has been shown (11)]; 9–12, serial dilutions of the recombinant plasmid pCp16 digested with *Hind*III/*Hind*II to generate a 246-bp fragment, including the whole repeat, used as a reference to titrate the number of repeat units [lane 9, 26 copies; lane 10, 132 copies; lane 11, 263 copies; lane 12, 1315 copies; similar titrations were done for BR1 and BR2 (not shown)]; 1, 5, and 6, blots of total DNA digested to completion with restriction enzymes with no recognition sites in the sequenced repeat units. These are *Alu* I/*Sau*3AI (lane 1) for BR1 and *Alu* I/*Rsa* I (lanes 5 and 6) for BR2 and BR6, respectively. Single digests with each of these enzymes gave similar results (not shown).

FIG. 3. Sequences of the repeat units cloned in pCp90 (BR1), pCp41 (BR2), and pCp16 (BR6) shown in the 5' → 3' direction. The constant parts that are homologous have been aligned, and nucleotides or amino acids in pCp41 and pCp16 that are identical to those of pCp90 are indicated with dashes. The subrepeated parts, which follow in the 3' direction, cannot be made homologous and are shown separately, divided into subrepeats. Also in pCp41 the sequence in the subrepeated part is complete but has been arranged so as to show the two types of subrepeats in separate rows. Insertion sites into the vector are shown with arrowheads. For pCp16 the left arrowhead shows the junction to the vector (insert 3', vector 5'), and the right arrowhead shows the junction to the remainder of the insert, which does not have BR repeat character. (Lower Center) Models of parts of the genes represented by the cloned inserts and the location of the inserts in relation to the repeat structure are shown. Subrepeats are shown as boxes (for BR2 of two different kinds) and unique parts are shown as single lines connecting the rows of boxes. For pCp16, only the repeat part of the clone is indicated. Here the junction to the vector is to the right and the rightmost 2/3 indicates the position of pCp16-7.

stretches of similarities. The subrepeated part consists of two five-codon repeats arranged in the order ababba (a = Thr-Arg-Ser-Gly-Leu, b = Arg-Pro-Glu-Arg-Pro) (Fig. 3). The coded composition is similar to that of pCp90 (Table 1).

The BR6 Insert. A plasmid was obtained containing an insert hybridizing to BR6. Part of the insert is described here

Table 1. Coded composition of common amino acids from repeats of BR1 (pCp90), BR2 (pCp41), and BR6 (pCp16)

	Composition, mol/100 mol of amino acid		
	pCp 90	pCp 41	pCp 16
Arg	16.9	23.1	22.4
Lys	14.3	9.2	7.5
Sum basic	31.2	32.3	29.9
Asp	1.3	0.0	11.9
Glu	3.9	7.7	26.9
Sum acidic	5.2	7.7	38.8
Ser	16.9	13.8	0.0
Thr	5.2	3.1	0.0
Sum ser + thr	22.1	16.9	0.0
Pro	19.5	12.3	13.4

as representing 204 bp of a total of 902 bp. This part extends from the 3' end of the insert and includes the subcloned pCp16-7 (Fig. 3). The remainder of pCp16 is not part of the typical BR repeat structure. The pCp16-7 was used to obtain the DNA blotting results presented in Fig. 2 (lanes 6–12). Both plasmids hybridized *in situ* to BR6 (Fig. 1).

The 204-bp fragment, minus an overlap of three bases at the beginning and the end, probably represents a whole repeat unit. This is because it can be made homologous with other published BR repeat units if joined at this overlap (Fig. 3). The assumption is also supported by results of Southern blots of partial and complete digests of genomic DNA, suggesting a repeat unit of about 200 bp (Fig. 2, lanes 7 and 8). Titration experiments indicated that the repeat is represented about 113 times (average of 86 and 139), corresponding to 23 kb of DNA (Fig. 2, lanes 8–12) probably in a single block (Fig. 2, lane 6). Because BR6 is likely to be smaller than BR2 (more rapidly migrating 75S RNA and translation product in NaDodSO₄ gels; ref. 9), the repeat should be representative for a considerable part of the gene if there is one gene per genome. If one considers that a block of about 20 kb was seen after simultaneous digestion with two restriction endonucleases that recognize four bases, the sites of which are not represented within the cloned sequence, it is unlikely that the repeat is divided among several genes per genome. The repeat contains a constant and a subrepeated part, the latter consisting of a three-codon combination represented 11 times with extensive variation between some of the individual subrepeats. Compared with other BR repeat units there are conserved sites in the constant part—i.e., all four -Cys-, -Met-Arg-, and -Arg-Phe- residues. The coded amino acid composition (Table 1) differs from BR1 and BR2 mainly in lack of serine and threonine and a high content of glutamic acid. At the nucleotide level, the unique part shows 38% and 43% homology with pCp90 and pCp41, respectively.

Phosphorylation Studies. The high contents of codons for serine and threonine in the BR1 and BR2 sequences and their absence in the BR6 sequence suggested that *P* might be used to neutralize at least some of the excess of positive charges in the BR1 and BR2 products (cf. ref. 32).

Glands were incubated with [γ -³²P]ATP, and the sister glands, with [³⁵S]methionine. Glands from induced animals and two types of controls were used. One control was animals under normal culture conditions (*cc*); the other was animals with only cellulose, parallel controls (*c*)—i.e., the same conditions as for induced animals (*g*) but without inducer. Total gland proteins were separated in composite gels. The ³²P label was in sp-Ia and sp-Ib but not in sp-Ic (Fig. 4).

The response to glycerol is complex, with decrease in BR2 activity and induction of BR6 when animals are taken from normal culture conditions to inducer medium (without food). The suppression of BR2 (and sp-Ib) may be due to starvation as such. The induction—i.e., the difference between induced animals (*g*) and parallel controls (*c*), which produced mainly sp-Ia—is thus simpler, involving only the addition of sp-Ic. In this latter comparison, the total production of secretion increases, probably due to the presence of metabolizable glycerol. The sp-Ic consists of two, probably allelic, forms, sp-Ic₁ and sp-Ic₂ (8). These also could be observed here, with sp-Ic₁ quantitatively dominant (Fig. 4).

The uptake of *P* into secretory protein is due to esterified *P* because alkaline phosphatase and pronase eliminate the incorporated ³²P (Fig. 5a). Controls showed that the phosphatase caused only slight degradation of sp-I components. Thin-layer electrophoretic analyses showed that the label co-migrates mainly with phosphoserine (Fig. 5b).

Quantitative determinations of alkali-labile *P* (characteristic for phosphoserine and phosphothreonine; ref. 21) in total secretory protein of glands from untreated animals showed a value of 630 μ mol of *P* per g of protein (Table 2). In agree-

FIG. 4. Separations in 2.1% polyacrylamide/1% agarose composite NaDodSO₄ gels (10) of salivary gland proteins from glycerol-induced animals (lanes *g*), parallel controls (lanes *c*) and normal culture controls (lanes *cc*). Parallel controls received a treatment identical to that of induced animals but without inducer, whereas culture controls were kept under normal culture conditions with food. Six glands from six animals of each kind were used for [³⁵S]methionine incorporation (lanes without primes) and the sister glands for [³²P]ATP incubation (lanes with primes). The six resulting samples were electrophoresed. The whole samples were used for *c* and *g* separations, but only 2/3 of each sample was used for the *cc* separations. (Left) Gel stained with Coomassie blue (C. bl.). (Right) Gel processed for fluorography to detect [³⁵S]methionine and for autoradiography to detect ³²P incorporation, indicated as ³⁵S and ³²P, respectively. The arrow shows the origin. Positions of sp-Ia, sp-Ib and sp-Ic are indicated. The sp-Ic fraction is complex, consisting of a slower migrating sp-Ic₁ and a faster sp-Ic₂ (8). The stained separations were impregnated with EN³HANCE and dried for fluorography to show [³⁵S]methionine uptake or dried for autoradiography to show uptake of ³²P.

ment with the predominance of sp-Ia and sp-Ib among the secretory proteins isolated, sp-I showed a somewhat higher content, corresponding to eight *P* atoms per 100 amino acids. In conjunction with the amino acid compositions, this suggests that about half of the serines are phosphorylated.

FIG. 5. *P* incorporation into secretory proteins. (a) Autoradiograph of separations in a 2% agarose/NaDodSO₄ gel is shown with ³²P-labeled sp-I isolated by Sepharose 6B-CL filtration (right lane), after pronase digestion (middle lane), and after digestion by alkaline phosphatase (left lane). Total secretion was also prepared and run in parallel to confirm positions of sp-Ia and sp-Ib (not shown). The gels were dried and autoradiographed. (b) Thin-layer electrophoretic separation in which hydrolysates of ³²P-labeled sp-I were separated with marker phosphothreonine [thr(P)] and phosphoserine [ser(P)]. Two different hydrolysates (1 μ l each) were applied (lane i) and (lane ii). These aliquots contained 265,000 and 208,000 cpm, respectively. In lane i the ser(P) spot contained 233,000 cpm and the thr(P) spot, 11,700 cpm; in lane ii the ser(P) spot contained 135,000 cpm and the thr(P) spot, 5,800 cpm. The *P*_i ran off the plate.

Table 2. Alkali-labile phosphate content of total secretory protein and chromatographically isolated sp-I

	<i>P</i> , mean $\pm$ SD*
Total secretory protein (μ mol of <i>P</i> /g of protein)	630 $\pm$ 34
sp-Ia + sp-Ib (μ mol of <i>P</i> /g of protein)	758 $\pm$ 28
sp-Ia + sp-Ib (<i>P</i> atoms/100 amino acids)	8.4 $\pm$ 0.3

**N* = 5.

DISCUSSION

The repeat units isolated here were similar to BR1 (BRb) and BR2 (BRc) units from other species in size and general structure, with a constant and a subrepeated part. Although the BR6 repeat showed a number of distinctive features in the constant part, some positions were identical to those in other repeats like the cysteines. The overall homology (about 40% with the unique parts of the BR1 and BR2 repeats) indicated that BR6 has a common origin with BR1 and BR2, although it has evolved more from them than BR1 and BR2 have from each other. The BR1 and BR2 repeats, although representing only about half of the genes, are relatively representative for the amino acid composition of isolated sp-Ia and sp-Ib, although differences exist (29). No amino acid analysis is available for sp-Ic. On the other hand, in this case the repeat is likely to be representative for most of the gene.

All BR repeats coded for about 30% basic amino acids. However, the BR6-coded sequence, because of an abundance of glutamic acid coded for amino acids with an excess of negative charges in contrast to the BR1 and BR2 repeats, with an excess of positive charges. The BR1 and BR2 sequences also coded for a high percentage of serine/threonine, the codons for which were absent in the BR6 repeat. These differences suggested that the requirement for neutralization in the BR1 and BR2 products might, at least partially, be fulfilled by phosphorylation, as confirmed by direct analyses. *P* was incorporated into sp-Ia and sp-Ib (translation products of BR1 and BR2) but not measurably in sp-Ic (the putative product of BR6). *P* was esterified with protein mainly as phosphoserine and to a minor extent as phosphothreonine. Quantitative determinations suggested that about half of the serines are phosphorylated—i.e., eight *P* atoms per 100 amino acids, an unusually high phosphorylation. It is interesting that Arg-Pro-Glu sequences in BR6 subrepeats have Arg-Pro-Ser or -Thr counterparts in BR1 and BR2. The latter, if phosphorylated, might functionally mimic the former.

Three BR6 inducers, galactose, ethanol, and glycerol, all decrease *P*_i in the hemolymph; glycerol, in the concentrations used here, decreases *P*_i from 15 mM to 5 mM (9). Because the haemolymph is about 50% of the weight of the animals (estimated by weighing larvae before and after exsanguination), this is a considerable loss of *P*_i. If glands can renew their secretion content once per day (2), it implies daily export of 10–15 μ g of secretory protein (10), most of which is sp-I. Because the *P* content for total secretory protein is about 0.6 nmol/ μ g of protein, the *P* loss per day could be 6–9 nmol. A 20-mg larva contains about 10 μ l of hemolymph, which after glycerol treatment contains *P*_i at about 5 nmol/ μ l, all in all about 50 nmol (9). An unchanged secretory protein export would thus deplete *P*_i rapidly in induced animals, which have, furthermore, no possibility of replenishing

their *P* supplies. Starving controls also have no *P* access. They are able to uphold the hemolymph *P*_i values, although they do not induce BR6 (9). This is possibly because the production of giant secretory proteins is reduced (Fig. 4) to mainly sp-Ia and/or because they are not exposed to the *P* binding action of the inducer. In any case the BR6 induction permits *P* to be retained for a longer time than if BR1 + BR2 protein had been produced in unchanged amounts.

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