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Oligomerization and Cell-Binding Properties of the Avian Reovirus Cell-Attachment Protein σC

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Avian reovirus protein σC, the viral cell-attachment protein, is a minor component of the outer-capsid shell of the viral particle that is synthesized in small amounts in infected cells. We cloned the σC-encoding ORF in vector pIL-2f, expressed it in *Escherichia coli,* and partially purified the resulting recombinant protein from inclusion bodies. Rabbit polyclonal antibodies raised against the recombinant protein specifically recognized the viral polypeptide in ELISA, immunoprecipitation, and Western blotting. To study the oligomerization capacity and cell-binding affinity of protein σC, the σC-encoding ORF was also expressed in chicken embryo fibroblasts (CEFs) and in reticulocyte lysates. In all three systems protein σC is expressed as a multimer with identical electrophoretic mobility to the naturally occurring protein. Cell-binding experiments show that both *in vitro* and *in vivo* expressed protein σC display affinity for CEF receptors, and this property is exclusively associated with the oligomeric form of the protein. The fact that incubation of CEF cells with the recombinant protein expressed in bacterial cells completely blocks the binding of purified reovirions indicates both that binding of this protein to cells is specific and saturable, and that reovirions and protein σC bind to the same class of cell receptor. Saturation binding experiments, performed with the recombinant protein expressed in *E. coli* and with purified reovirions, showed that the number of cellular receptor sites (CRSs) for avian reovirus S1133 is 1.8×10^4 per CEF cell, whereas the number of cellular receptor units (CRUs) for σC is 2.2×10^5 per CEF cell. These results are consistent with previous reports on the binding of mammalian reoviruses.

Key Words: avian reovirus; protein σC; oligomerization; cell binding.

INTRODUCTION

Avian reovirus strain S1133 belongs to one of the nine genera of the family *Reoviridae,* that is, *Orthoreovirus,* which also includes the well-studied mammalian reovirus (Armstrong *et al.,* 1984). Both groups of viruses share morphological and biochemical characteristics (Spandidos and Graham, 1976). However, the lack of hemagglutinin, the ability to induce cell fusion, and a different host cell range separate avian reoviruses from their mammalian counterparts (Ni and Ramig, 1993; Theophilos *et al.,* 1995; Wilcox *et al.,* 1982). Avian reoviruses possess a double capsid shell enclosing 10 segments of dsRNA that code for at least 10 primary translation products, which can be grouped into three size classes (λ, μ, and σ) according to their mobility in SDS–PAGE (Martínez-Costas *et al.,* 1997; Spandidos and Graham, 1976; Varela and Benavente, 1994).

A critical event in viral pathogenesis is the interaction between viral cell-attachment proteins and host cell receptors. This interaction is a specific process which leads to the internalization of the virus and finally to the infection of the host cell. The σC protein of avian reovirus is a minor outer-capsid protein encoded by the third open reading frame (ORF) of the S1 genome segment (Liu and Giambrone, 1997; Shapouri *et al.,* 1995; Varela and Benavente, 1994). This protein has been identified as the cell-attachment protein because it is the only viral polypeptide present in extracts of infected cells that is able to bind specifically to chicken embryo fibroblasts (CEFs) (Martínez-Costas *et al.,* 1997). Evidence for the multimeric nature of the protein σC present in reovirions and in extracts of infected cells has been reported (Martínez-Costas *et al.,* 1997; Shapouri *et al.,* 1996). However, the differences in electrophoretic behavior between protein σC transiently expressed in COS-7 cells and that present in reovirions (Shapouri *et al.,* 1996) led these authors to suggest that multimerization of protein σC is controlled by virus-associated factor(s).

Protein σC has been associated with neutralization of virus infectivity both in CEFs and in Vero cells (Wickramasinghe *et al.,* 1993), and a role of this protein in the virus-induced cell fusion process has also been suggested (Theophilos *et al.,* 1995; Meanger *et al.,* 1999), but direct evidence for syncytium-inducing activity is still lacking. Protein σC has been expressed in *Escherichia coli* as a recombinant protein fused to the maltose-binding protein (MBP) (Shapouri *et al.,* 1996). SDS–PAGE analysis of the expressed σC-MBP showed that only a very small fraction of the fusion construct was oligomeric (Shapouri *et al.,* 1996; our unpublished observations).

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Cell-binding studies performed with biotin-labeled σC-MBP suggested that σC was capable of attaching to Vero cell monolayers in its multimeric form, and that its binding caused a reduction in virus infectivity (Shapouri *et al.,* 1996). However, the low oligomerization capacity of this construct, the fact that incubation of σC-MBP with Vero cells did not completely inhibit infectivity (Shapouri *et al.,* 1996), and our recent finding that the MBP moiety itself, when expressed in *E. coli,* displays some affinity for CEF monolayers (data not shown), suggest that σC-MBP is not an adequate construct for this type of cell-binding experiment, and that experiments with nonfused σC are required.

In the work reported here, we cloned the ORF encoding σC, expressed it in *E. coli,* partially purified the resulting recombinant protein σC from inclusion bodies, and raised antibodies against the partially purified protein. We also expressed the ORF in CEFs and in reticulocyte lysates, and compared the protein expressed in the three systems with the naturally occurring protein as regards oligomerization and attachment to CEF receptors. In addition, the partially purified recombinant σC from *E. coli* was used, along with purified avian reovirions, to calculate the number of CRUs (cellular molecules recognizing one cell-attachment protein) and CRSs (cellular structures composed of one or more CRUs) per cell.

RESULTS

Cloning and expression of the gene encoding avian reovirus protein σC

Previous results have shown that avian reovirus S1133 σC protein is encoded by the 3′ proximal ORF of the S1 genomic segment (Liu and Giambrone, 1997; Shapouri *et al.,* 1995). To be able to express large amounts of protein σC, its ORF was cloned into the expression vector pIL-2f (Dueñas *et al.,* 1994) as described under Materials and Methods. In this vector the cloned gene is under the control of the *trp* promoter and uses the T4 terminator (Dueñas *et al.,* 1994; Nichols and Yanofsky, 1983). The *E. coli* strain W3110 was transformed with the recombinant plasmid and, after screening, the positive clones were grown and the plasmid isolated and sequenced. Protein σC was expressed by induction with 3-β-indoleacrylic acid in M9 minimal medium.

Analysis by SDS–PAGE and Western blotting, using polyclonal antibodies against purified reovirions (Fig. 1A), showed that protein σC was not synthesized in bacteria transformed with plasmid pIL-fiber (lane 1) (which contains an insert coding for adenovirus fiber protein). However, possibly as a result of "leaky" transcription, both uninduced bacteria harboring the plasmid pIL-σC (lane 2) and induced cells (lane 3) produced the σC polypeptide. When induced bacteria expressing the σC polypeptide were disrupted and the sample was centrifuged, the σC polypeptide could not be detected in the supernatant fraction (lane 4), but it appeared in the pellet fraction (lane 5), suggesting that the expressed σC protein was assembled into inclusion bodies.

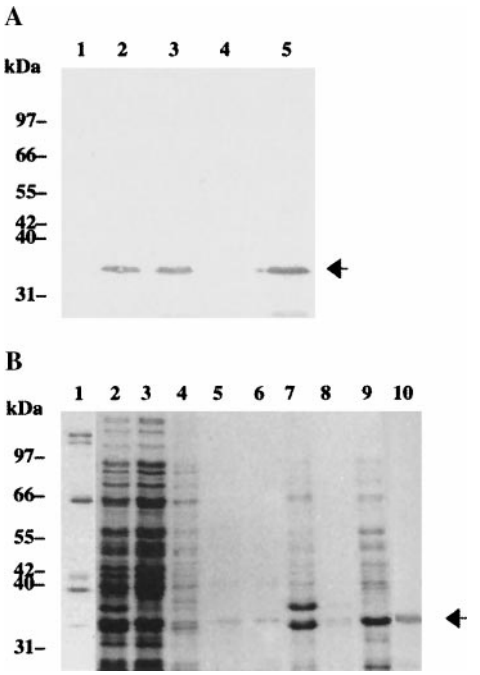

FIG. 1. Expression of the avian reovirus σC protein in *Escherichia coli.* An overnight culture of pIL-σC bacteria in LB was inoculated into 100 ml of supplemented M9 minimal medium. After 1 h of incubation, protein expression was induced with 3-β-indoleacrylic acid. At about 12 h postinduction the culture was processed as described under Materials and Methods. (A) Western blot analysis, using polyclonal antibodies against purified avian reovirions, of the following samples: induced bacteria expressing adenovirus fiber protein (lane 1), uninduced (lane 2), and induced bacteria (lane 3) harboring pIL-σC, supernatant (lane 4), and pellet (lane 5) after centrifugation of sonicated induced pIL-σC bacteria. (B) Samples of the different purification steps (lanes 2–9; see below) and of purified reovirions (lane 1) were analyzed by SDS–PAGE. After electrophoresis, the gel was fixed and then stained with Coomassie brilliant blue. Lane 2, total biomass of induced pIL-σC bacteria; lane 3, supernatant of pIL-σC bacteria after disruption in a French press; lane 4, supernatant of Triton X-100 wash of inclusion bodies; lane 5, PBS wash of inclusion bodies; lane 6, supernatant of 2 M urea washing; lane 7, pellet after solubilization with 8 M urea; lane 8, pellet obtained after centrifugation of the sample subjected to dialysis; lane 9, final supernatant of the dialysed sample; lane 10, Western blotting analysis of the sample shown in lane 9 using anti-reovirion antibodies. Positions of the relative mol wt markers are indicated on the left, and the position of the σC band is indicated with an arrow at the right of the figure.

Partial purification of the recombinant σC protein

Once expression of protein σC in *E. coli* had been demonstrated, we undertook large-scale culture of the transformed bacteria, to obtain partially purified σC in larger quantities. For this purpose, 15-ml overnight cultures of pIL-σC bacteria in LB were inoculated into 100 ml of minimum M9 medium and induced with 3-β-indoleacrylic acid as earlier. Nine to 13 h postinduction the

culture was processed as described under Materials and Methods. The quality of the material recovered at each step of the purification procedure is shown in a Coomassie brilliant blue-stained gel after SDS–PAGE (Fig. 1B). Protein σC was detected in both the total biomass of induced pIL-σC *E. coli* culture (lane 2) and in the supernatant obtained after disruption of these bacteria in a French press (lane 3). After incubation of the inclusion bodies in washing solution, many cytoplasmic proteins and a small amount of σC were released (lane 4). Surprisingly, protein σC was the major constituent among the polypeptides that were released after washing the inclusion bodies with PBS (lane 5). A wash with 2 M urea failed to solubilize the inclusion bodies, but rather removed other cellular proteins and also some σC (lane 6). After incubation with 8 M urea and subsequent centrifugation, some bacterial proteins precipitated (lane 7). The resulting supernatant was subjected to dialysis followed by a final centrifugation step, which removed insoluble proteins that pelleted (lane 8). The final supernatant (lane 9) contained a major soluble protein migrating to the same position as the reovirion σC (lane 1). A Western blot analysis of the sample in lane 9, using anti-S1133 polyclonal antibodies (lane 10), confirmed the identity of the major soluble protein as the avian reovirus protein σC.

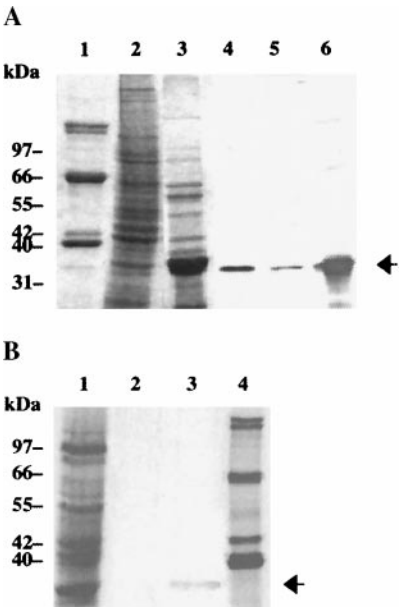

FIG. 2. Characterization of the polyclonal anti-σC antibodies. (A) By Western blotting: samples of purified avian reovirus (lanes 1 and 4), cytoplasmic extracts of avian reovirus-infected CEF cells (lanes 2 and 5), and the partially purified σC expressed in bacteria (lanes 3 and 6) were resolved by SDS–PAGE, and the gel was then either stained with Coomassie brilliant blue (lanes 1–3) or subjected to immunoblotting analysis (lanes 4–6) with the polyclonal antibodies raised in rabbits against the sample shown in lane 9 of Fig. 1B. (B) By immunoprecipitation: the supernatant obtained after incubation of [^{35}S]methionine-labeled inclusion bodies in washing solution (lane 1) was subjected to immunoprecipitation with preimmune serum (lane 2) or with anti-σC antiserum (lane 3). These samples, as well as purified radiolabeled reovirions, were analyzed by SDS–PAGE and autoradiography.

Preparation and characterization of polyclonal anti-σC antibodies

The sample shown in lane 9 of Fig. 1B was used as antigen to raise polyclonal antibodies in a rabbit. The specificity and affinity of the polyclonal antiserum was evaluated in three different ways: ELISA, Western blotting, and immunoprecipitation.

ELISA was performed as described under Materials and Methods. The results indicate high specificity for the σC protein isolated from bacteria, with a maximal titer at a dilution of 1/6400; even at a dilution of $1/8 \times 10^6$ the signal was still significant. No reaction was observed when the target antigen was the recombinant adenovirus fiber protein expressed in the same way or BSA. The preimmune serum did not react with any of these samples.

For Western blotting (Fig. 2A), samples of the purified reovirions (lane 1), of extracts of S1133-infected CEF (lane 2), and of the partially purified protein expressed in bacteria (lane 3) were boiled in Laemmli sample buffer and then analyzed by SDS–PAGE. The polypeptides in the gel were subsequently transferred to a nitrocellulose filter, and the filter was probed with our anti-σC antiserum. In all three samples (lanes 4–6) the antibodies recognized a single polypeptide with identical electrophoretic mobility to the reovirion σC polypeptide (lane 4).

For immunoprecipitation (Fig. 2B), the first wash of the inclusion bodies obtained from bacterial cells that had been metabolically labeled with ^{35}S-methionine (Fig. 2B, lane 1) was used, since this fraction contains, in addition to σC, several other prominent bacterial proteins serving as negative controls to demonstrate the specificity of the antibodies. Although the preimmune serum did not recognize any of the proteins present in the extract (lane 2), the polyclonal anti-σC antibodies specifically recognized one protein (lane 3) that comigrated with the σC polypeptide present in reovirions (lane 4). Taken together, these results confirm both that the recombinant protein expressed in bacteria is avian reovirus σC and that the polyclonal antibodies raised in rabbit against the recombinant protein are able to recognize the viral polypeptide in ELISA, Western blotting, and immunoprecipitation.

Oligomerization

It has been established that the protein σC present in both virions and extracts of infected cells is a multimer in its native state, and electrophoretic analysis of the monomeric and oligomeric forms of this protein have suggested that the multimer is made up of three monomer units (Martínez-Costas *et al.,* 1997). However, the fact that the oligomeric form of σC, when transiently ex-

pressed in COS-7 cells, displays different electrophoretic behavior to the protein present in reovirions (Shapouri *et al.,* 1996) led these authors to suggest that oligomerization of avian reovirus σC protein is controlled by virus-associated factor(s).

To characterize the functional properties of the σC protein expressed in bacteria, and to investigate whether the multimerization of this protein requires viral factors, a comparative electrophoretic analysis of the recombinant σC from *E. coli* and the native protein was performed under both dissociating and nondissociating conditions. Specifically, the sample shown in lane 4 of Fig. 1B (the first wash of the inclusion bodies), and samples of both purified avian reovirions and extracts of S1133-infected CEF, were incubated for 5 min in Laemmli sample buffer at either 37°C (to retain maximal oligomerization) or at 90°C (for oligomer denaturalization). A subsequent analysis of the samples, by SDS–PAGE followed by anti-σC Western blotting (Fig. 3A), indicated that recombinant σC expressed in bacteria is an oligomer in its native state (pIL-σC, lane U) that displays identical electrophoretic behavior to the protein σC present in reovirions (S1133) and in lysates of virus-infected cells (S1133-CEF), whether as a monomer (lanes B) or an oligomer (lanes U). This finding demonstrates that the protein σC expressed in bacteria is an oligomer in its native state, and suggests that correct oligomerization of σC does not require any virus-associated factor. To confirm that this is the case, we investigated the oligomerization capacity of σC expressed *in vitro* and in CEF cells.

To express the protein in CEF, the σC-encoding ORF was inserted into the pCIneo vector and the recombinant plasmid along with the translation-enhancing plasmid pAdVAntage (Promega, Madison, WI) were lipofected into monolayered CEF. A Western blot analysis of extracts of the transfected cells (Fig. 3B) showed that protein σC was synthesized when the cells were lipofected with both constructs (lanes 4 and 7), but not when either the pAdVAntage construct (lanes 2 and 5), the pCIneo-σC construct (lanes 3 and 6), or the lipofectin (data not shown) were omitted from the transfection assay. This experiment also showed that under both nondenaturing (Fig. 3B, lane 4) and denaturing conditions (Fig. 3B, lane 7) the protein σC transiently expressed in CEF displays similar electrophoretic mobility to the protein σC present in reovirions (Fig. 3B, lanes 1 and 8, respectively). Similar results were obtained with the protein σC synthesized in reticulocyte lysates by *in vitro* expression of the σC-encoding ORF (Fig. 3C), although in this case only a small fraction of the σC protein synthesized *in vitro* was in the oligomeric form (Fig. 3C, lane 4).

Taken together, our results clearly demonstrate both that protein σC is an oligomer in its native state and that it is able to oligomerize correctly in the absence of any viral factor.

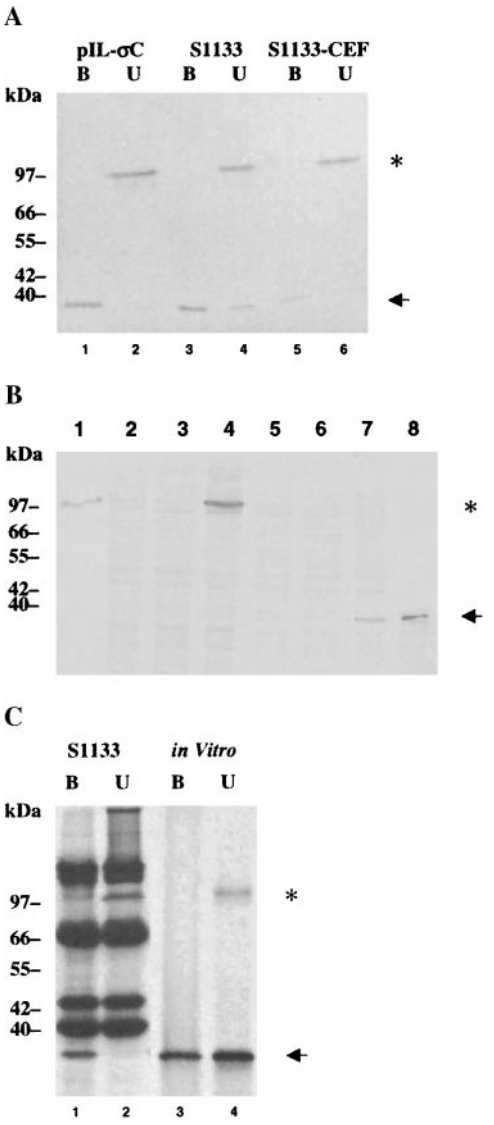

FIG. 3. Multimerization capacity of protein σC from different sources. (A) The sample shown in lane 4 of Fig. 1B (pIL-σC), purified avian reovirions (S1133), or cytoplasmic extracts of infected cells (S1133-CEF) were either boiled (B) or incubated at 30°C (U) in Laemmli sample buffer. Polypeptides were separated by SDS–PAGE, transferred to a nitrocellulose filter, and subjected to Western blotting analysis with anti-σC serum. (B) CEF monolayers were transfected with the plasmid pCIneo-σC (lanes 2 and 5) or with the plasmid pAdVAntage (lanes 3 and 6), or were cotransfected with both plasmids (lanes 4 and 7), then incubated overnight at 30°C. Cytoplasmic extracts of the transfected cells (lanes 2–7), as well as samples of purified avian reovirions (lanes 1 and 8), were mixed with an equal volume of 2× Laemmli buffer. Samples were incubated at 30°C (lanes 1–4) or boiled at 90°C for 5 min (lanes 5–8), and then subjected to SDS–PAGE and Western blotting analysis with anti-σC polyclonal antibodies. To ensure clear visualization of the band corresponding to the σC monomer, four times as many reovirions were loaded in lane 8 as in lane 1. (C) ^{35}S-labeled protein σC was synthesized in reticulocyte lysates as described under Materials and Methods. Samples of purified ^{35}S-labeled reovirions (lanes 1 and 2) and of the *in vitro* translation products (lanes 3 and 4) were incubated for 5 min with an equal volume of Laemmli sample buffer either at 90°C (lanes 1 and 3) or at 30°C (lanes 2 and 4). Samples were then analyzed by SDS–PAGE and autoradiography.

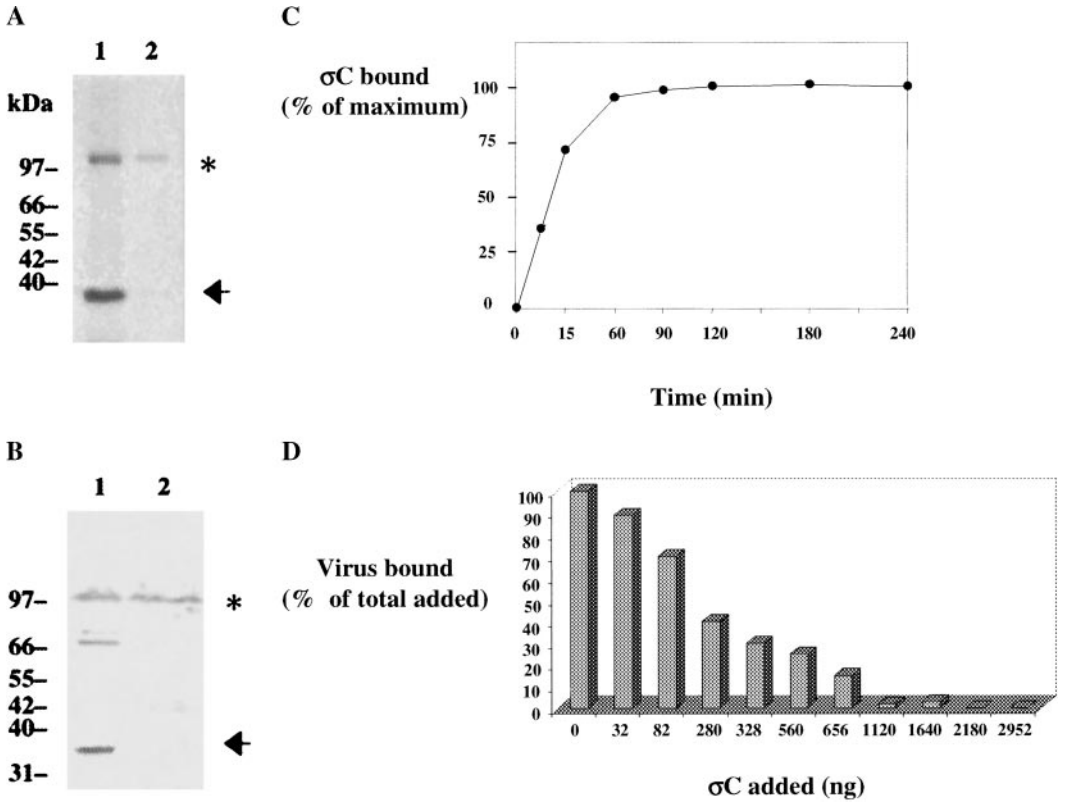

FIG. 4. Binding assays. (A) A reticulocyte lysate containing ^{35}S-labeled protein σC (lane 1) was incubated for 1 h at 4°C with a CEF monolayer and, after extensive washing of the monolayer with PBS, the cells were lysed, the nuclei were pelleted, and the resulting supernantant (lane 2) was analyzed by SDS–PAGE under nondissociating conditions. (B) A sample similar to that shown in lane 9 of Fig. 1B, containing partially purified σC from bacterial cells, was subjected to nondissociating SDS–PAGE and anti-σC Western blot analysis either before (lane 1) or after (lane 2) incubation with a CEF monolayer. Positions of the σC oligomer and monomer bands are marked at the right of the figure with an asterisk and an arrow, respectively. Positions of the relative mol wt markers are indicated on the left. (C) Confluent monolayers of CEF grown in 24-well plates (6.5×10^6 cells per well) were overlaid with 200 μl of binding buffer containing approximately 300 ng of purified ^{35}S-labeled σC trimers produced in *E. coli* and incubated at 20°C for the period of time indicated at the bottom of the figure. After extensive washing of the cell monolayers with cold PBS, the cell-associated radioactivity was measured and the results expressed as percentage of maximum binding, which was reached at 120 min. Each point represents the means ± SD of three wells. (D) Increasing amounts of the partially purified protein σC expressed in bacteria were incubated on ice for 90 min with monolayers of 6.5×10^5 CEFs in 24-well plates in the presence of 200 μl of binding buffer. The monolayers were washed and a constant amount of ^{35}S-labeled avian reovirus (60,000 cpm, 4 μg) was added to each well and incubated for 90 min on ice. Radioactivity was determined in wash and cell fraction. Each bar represents the mean of four experiments.

Cell binding

We next investigated the capability of σC expressed in the different systems to bind to monolayers of CEFs. First, the binding assay was performed with a radioactive sample of reticulocyte lysates that had been programmed with the σC mRNA (Fig. 4A, lane 1). As can be seen from lane 2 of Fig. 4A, *in vitro* synthesized σC binds to CEF receptors, but only the oligomeric form (not the monomeric form) showed binding activity, despite the fact that most of the σC present in the reticulocyte extract was in the monomeric form (Fig. 4A, lane 1). We next performed the binding assay with recombinant σC from *E. coli.* For this assay we used the sample shown in lane 9 of Fig. 1B, since it contains three different oligomeric forms of protein σC (Fig. 4B, lane 1), allowing us to assess which of these forms possesses binding activity. An anti-σC Western blotting analysis of extracts of monolayer CEFs that had been incubated with the sample shown in lane 9 of Fig. 1B revealed that the recombinant σC protein synthesized in bacterial cells binds to CEF monolayers. The results of this experiment also showed that only the 100-M_r form of the protein, not the 70- or 38-M_r forms, binds to CEF receptors (Fig. 4B, lane 2). A similar result was obtained when a CEF monolayer was incubated with an extract containing the σC protein transiently expressed in CEF cells (data not shown). These results demonstrate that the 100-kDa oligomeric form of protein σC, whether synthesized *in vitro* or *in vivo,* whether in eukaryotic or prokaryotic cells, has similar biological activity to the naturally occurring protein.

A kinetic study of the attachment of the partially purified protein σC from bacterial cells revealed that binding of σC to cell receptors occurs linearly during the first 15 min and reaches a plateau at about 60–90 min. A similar

experiment performed with avian reovirus particles (Grande and Benavente, 2000) showed that maximum attachment of the virus occurs at 90–120 min. The binding of recombinant σC to the cell monolayer was specific, since it caused inhibition of the attachment of purified reovirions to host-cell receptors, in a dose-dependent manner (Fig. 4D). The attachment of purified reovirions was not inhibited when an irrelevant protein (recombinant adenovirus fiber protein) was used instead of protein σC (data not shown). The fact that recombinant σC was able to completely block binding of an excess of reovirions to cell receptors indicates both that attachment of this protein to CEFs is specific and saturable, and that protein σC and reovirions bind to the same class of cell receptors.

Saturation of cell receptors by recombinant protein σC and by reovirions

To estimate the number of σC receptors present on the surface of CEF cells, saturation binding experiments were performed with both the partially purified protein σC expressed in bacteria and with purified reovirions. To this end, increasing amounts of ^{35}S-labeled σC were added to cell monolayers until saturating levels were reached. A plot of bound σC versus free σC (Fig. 5A) gave a typical monophasic saturation binding curve. When the data were replotted by the method of Scatchard (Fig. 5A, inset) (Scatchard, 1949), a straight line was obtained, suggesting that a single class of specific noncooperative cellular attachment sites mediates binding of protein σC to CEFs. We estimated that there are about 2.2×10^5 cellular receptor units (CRUs) per cell for protein σC (assuming that σC is a trimer with a molecular mass of 102 kDa). A similar study performed with ^{35}S-labeled purified avian reovirions (Fig. 5B) indicated that there are approximately 1.8×10^5 cellular receptor sites (CRSs) per cell for the avian reovirus S1133.

DISCUSSION

Protein σC is the cell-attachment protein mediating binding of avian reovirus to the host cell (Martínez-Costas *et al.,* 1997; Shapouri *et al.,* 1996). This protein is functionally and structurally related to the mammalian reovirus protein σ1, and both play multifaceted roles in reovirus infection (Lee *et al.,* 1998; Martínez-Costas *et al.,* 1997; Meanger *et al.,* 1999; Shapouri *et al.,* 1996; Theophilos *et al.,* 1995). It has been shown that the protein present in virions and in extracts of S1133-infected CEFs is an oligomer, likely a homotrimer, and that only the oligomeric form of this protein displays cell-binding activity (Martínez-Costas *et al.,* 1997). Characterization of protein σC at the molecular level, to improve understanding of its structure and function, has been hindered by the difficulty of obtaining sufficient amounts of pure protein σC. Shapouri and coworkers (1996) tried to overcome this problem by expressing σC in bacteria as an MBP-σC fusion protein, thus facilitating purification of the recombinant protein. However, the introduction of an MBP moiety at the amino-terminus end of protein σC greatly reduced the capacity of the viral protein both to multimerize and to bind to cell receptors. This might also be the reason why incubation of an excess of MBP-σC with Vero cells did not cause complete abolition of avian reovirus infectivity. These results, and our recent finding that MBP itself, when expressed in bacteria, displays some binding to CEF monolayers (data not shown), suggest that MBP is not an adequate moiety for use in experiments of this type, and accordingly we decided to express σC in bacteria as a nonfusion protein.

The induction of pIL-σC bacterial cells resulted in expression of protein σC in insoluble form, and we therefore set up a protocol for its solubilization and purification from inclusion bodies. Following this protocol, which included successive incubations of the inclusion bodies with washing solution, PBS, 2 M urea, and 8 M urea, and a final dialysis of the 8 M urea wash, we were able to isolate a fraction in which protein σC was the major protein present (Fig. 1B, lane 9). This fraction proved to be good material for both antibody preparation and protein characterization. A comparative Western blot analysis of the oligomeric status of protein σC in both this fraction and the supernatant resulting from the incubation of the inclusion bodies with washing solution (Fig. 1B, lane 4) showed that the 8 M urea treatment caused denaturalization of a fraction of the σC oligomer (compare lane 2 of Fig. 3A with lane 1 of Fig. 4B), to yield a mixture of three different σC forms (Fig. 4B, lane 1). The 38-M_r form corresponds to monomeric σC, whereas the 70- and 100-M_r forms are likely dimeric and trimeric σC, respectively. The binding assay demonstrated that only the 100-M_r form, but not the 38- or 70-M_r forms, showed binding affinity for a CEF monolayer (Fig. 4B, lane 2). A similar result was obtained with the *in vitro* translated σC, and with the protein synthesized in CEFs, although in these cases the 70-M_r form was not present in the starting samples (Fig 4A, data not shown). The 100-M_r form of the protein σC synthesized in all three systems displayed identical electrophoretic mobility to the σC protein present in both reovirions and extracts of infected cells, suggesting that it represents the mature and functional form of the protein. Taken together, these results suggest that trimeric σC has binding affinity for CEF receptors and that this activity is not shared by the dimer or the monomer. Our results also demonstrate that the recombinant σC protein expressed in bacteria is functionally indistinguishable from the naturally occurring protein.

The fact that a significant portion of the σC protein expressed in bacterial cells remains as a functional oligomer after incubation of the inclusion bodies with 8 M urea indicates that this oligomer has a very stable

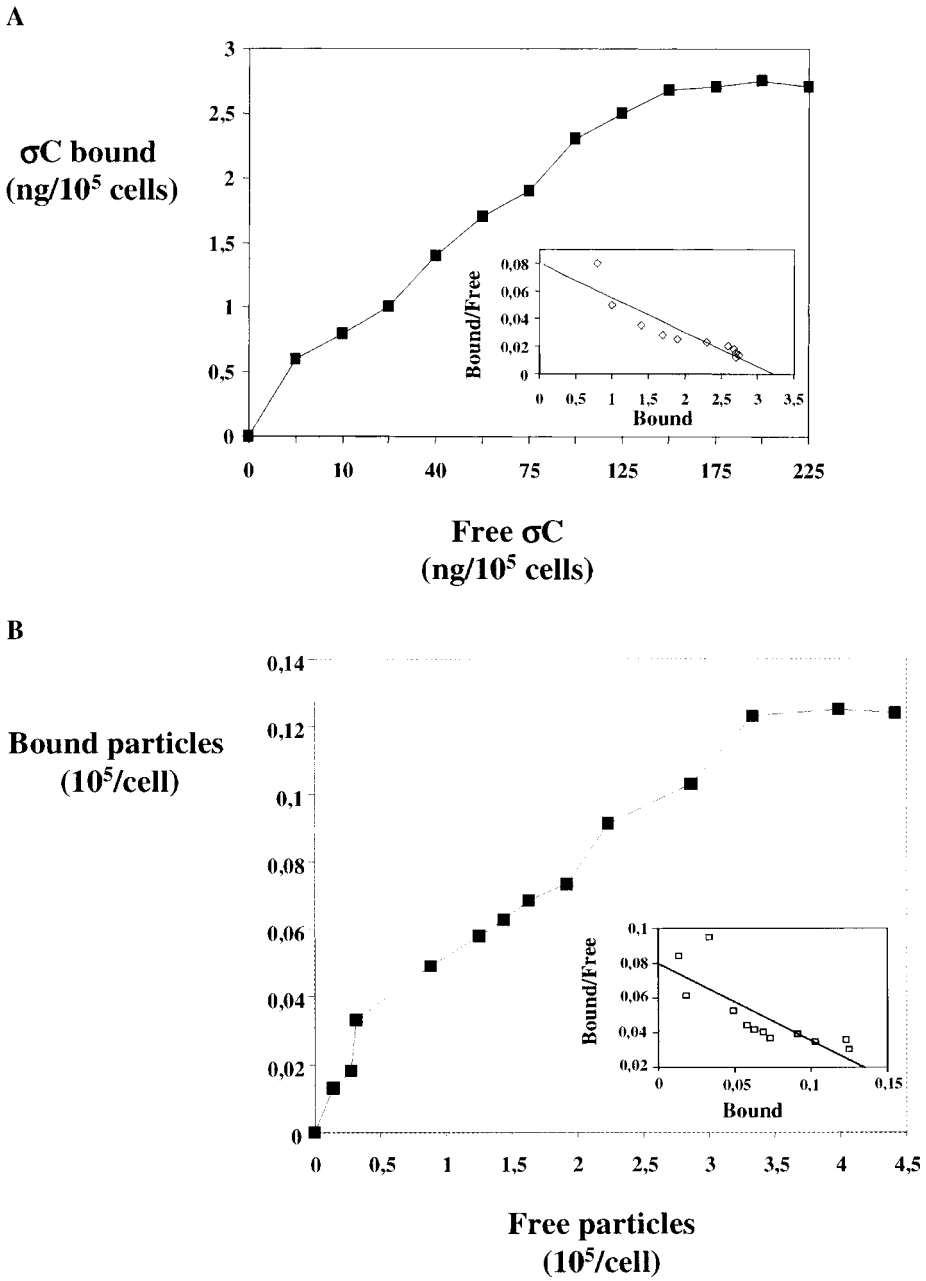

FIG. 5. Kinetics and saturability of binding of protein σC to CEFs. Increasing amounts of ^{35}S-labeled σC (A) and of ^{35}S-labeled reovirions (B) were added in 200 μl of binding buffer to confluent monolayers of CEFs in 24-well plates (6×10^5 cells per well) and incubated for 90 min on ice. Cell-associated radioactivity was determined after unbound σC had been washed off. Specific binding was calculated after subtracting the nonspecific binding observed in duplicate wells incubated in the presence of an excess of unlabeled purified protein σC and avian reovirions, respectively. Inset: Scatchard analysis of binding data. Each point represents the mean of three to five separate experiments.

conformation. Its stability is probably related to the heptapeptide repeat pattern found in the σC N-terminal region of all avian reovirus isolates examined to date (Liu and Giambrone, 1997; Shapouri *et al.,* 1995). A heptapeptide repeat with hydrophobic amino acids occupying positions a and d has been associated with the coiled-coil conformation found in many stable oligomeric proteins (Lupas, 1996), including the mammalian reovirus cell-attachment protein σ1 (Bassel-Duby *et al.,* 1985; Duncan *et al.,* 1990; Furlong *et al.,* 1988). This structural pattern is responsible for trimerization of protein σ1, for the stability of its oligomer, and for the incorporation of this protein into mammalian reovirions (Leone *et al.,* 1991a,b; Strong *et al.,* 1991). The fact that both the mammalian and avian reovirus cell-attachment oligomers show marked resistance to urea denaturalization contributes to support the notion that the two polypeptides are structurally and functionally related, as has been suggested previously (Lee and Gilmore, 1998; Liu and Giambrone, 1997; Shapouri *et al.,* 1996; Schnitzer, 1985;

Schnitzer *et al.,* 1982; Varela and Benavente, 1994; Wickramasinghe *et al.,* 1993).

The polyclonal antibodies raised in rabbits against the σC protein expressed in bacteria show a strong affinity for σC, and are able to recognize both the naturally occurring σC and the recombinant protein expressed in bacterial cells, in ELISA, Western blotting, and immunoprecipitation. We expect that these antibodies will be of great value in the near future for further study of the structure, properties, and activities of the poorly characterized avian reovirus σC protein.

Shapouri and coworkers (1996) expressed protein σC in COS-7 cells and studied its multimerization properties by analyzing its SDS–PAGE mobility under nondissociating conditions. They found that, whereas the virion-associated σC protein migrated in SDS–PAGE gels to a position of 70 kDa, the COS-7-expressed σC protein displayed a molecular mass of 200 kDa. This finding prompted the authors to suggest that some virus-associated factor(s) may control the multimerization of this protein. Our findings that protein σC, whether expressed in prokaryotic or eukaryotic cells, exhibits identical electrophoretic behavior to the naturally occurring protein clearly demonstrate that proper multimerization of protein σC can take place in the absence of any virus-associated factor(s). Furthermore, our finding that the *in vitro* synthesized protein σC has oligomerization capacity indicates that multimerization of σC does not require the presence of any intact cell structure, such as the cytoskeleton.

The σC protein expressed in bacteria efficiently blocks attachment of the virus to CEFs, suggesting both that recombinant σC and avian reovirions bind to the same class of cell receptor and that binding of σC to CEF receptors is specific and saturable.

The availability of biologically active σC prompted us to investigate the number of σC-receptor sites on CEFs. Our results show that binding of σC to CEF monolayers was specific and saturable, indicating the presence of only one class of cellular receptors for protein σC in CEFs. Scatchard plots of the saturation-curve data (Scatchard, 1949) indicated the presence of 1.8×10^4 CRSs per cell for avian reoviruses and 2.2×10^5 CRUs per cell for σC. These figures suggest that the avian reovirus receptors are minor components of the cell membrane and are in agreement with previous reports on the binding of mammalian reovirus: previous estimates include $2–8 \times 10^4$ sites per cell in several cell types (Epstein *et al.,* 1984), $8.6 \times 10^4–1.0 \times 10^5$ sites per cell in L cells and $1.2–1.4 \times 10^5$ sites per cell in HeLa cells (Gentsch *et al.,* 1984), and $3–5 \times 10^5$ sites per cell in L cells (Armstrong *et al.,* 1984). The number of receptors for other naked viruses (like picornavirus) has been calculated to be in the range of $10^4–10^6$ sites per cell (Lentz, 1990).

For adenovirus, another complex nonenveloped animal virus (which, like reovirus, has a fibrous protein at the 12 vertices of the icosahedral capsid), the number of fiber-protein-receptors per HeLa cell has been estimated to be about 10^5, but the number of receptors for intact adenovirus particles is only about 4000–6000 (possibly up to 10^4) (Persson *et al.,* 1985; Philipson *et al.,* 1968). This difference in the ratio of CRUs to CRSs, which for adenovirus is about 10–20 and for avian reovirus about 12, could be attributable to binding of the virus cell-attachment protein to cell membrane components inaccessible to the viral particle, or to the fact that binding of a reovirion to a particular cell receptor may sterically hinder the access of other viral particles to nearby receptors.

We hope that the availability of both specific anti-σC antibodies and large quantities of the avian reovirus σC protein (produced in bacteria, with similar conformation and cell-binding properties to the naturally occurring protein) will allow further structural and functional studies of this cell-attachment protein, and will facilitate the characterization and identification of the avian reovirus cell receptor.

MATERIALS AND METHODS

Cells and virus

Primary CEF cultures were prepared from 9- to 10-day-old chicken embryos and grown in medium 199 supplemented with 10% tryptose phosphate broth and 3.5% fetal bovine serum (all from Gibco BRL, Rockville, MD). Avian reovirus strain S1133 was grown in confluent monolayers of primary CEF cultures. Conditions for virus propagation, titration, and purification, and for metabolic radiolabeling of the virus, have been described previously (Grande and Benavente, 2000; Martínez-Costas *et al.,* 1995).

Cloning, sequencing, and expression of the ORF encoding σC

cDNA corresponding to the S1 genomic segment of avian reovirus was synthesized from messenger RNA recovered from infected cells after phenol-chloroform extraction followed by precipitation with potassium acetate and ethanol. The primer ATA ACC AAT CCC AGT ACG, complementary to the 3′ end of the s1 mRNA, was used at a final concentration of 5 ng/μl in the standard reverse transcription reaction catalyzed by AMV reverse transcriptase (Promega, Madison, WI). The synthesized cDNA was amplified by PCR using the following primers: (1) 5′-GGG CCTAGG T ATCGAAT ATG GCG GGT CTC AAT CCA TCG CAG-3′ and (2) 5′-GC GGATCC TTA GGT GTC GAT GCC GGT ACG CAC-3′. The first of these primers is identical to the 5′ end of the third ORF of the S1 gene segment, which also includes the sequences for restriction enzymes *Avr*II and *Cla*I. The second primer is complementary to the 3′ end of the same segment, and includes the sequence for *Bam*HI.

The amplified cDNA segment was cloned into the expression vector pIL-2, derived from plasmid pIL-2f (Dueñas *et al.,* 1994), which was digested with the restriction enzymes *Cla*I and *Bam*HI to release an insert that has been fused to a sequence coding for a 26-amino-acid N-terminal fragment of human interleukin-2.

Plasmid DNA was isolated using a Wizard Minipreps DNA purification system (Promega). DNA sequence analysis was done by the dideoxynucleotide chain termination method with the 5′ and 3′ primers described earlier (Sanger *et al.,* 1977), Sequenase version 2.0, and Redivue [α-^{35}S]dATP (Amersham Pharmacia Biotech, Piscataway, NJ).

Overnight cultures of recombinant *E. coli* strain W3110 gown in LB medium supplemented with 100 mg/ml of ampicillin were used as starting material for production of protein σC. Aliquots of this culture were inoculated into supplemented medium M9 (1% M9 salts solution, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2% casein hydrolysate, 0.5% glucose, and 100 mg/ml of ampicillin) to yield OD = 0.2. After 1 h of incubation at 37°C, the culture was supplemented with 20 μg/ml of 3-β-indoleacrylic acid (IA) (Sigma Chemical Co., St. Louis, MO) and grown for an additional 9–13 h. For radiolabeling of expressed σC protein, 0.5 mCi of Redivue Promix ^{35}S-cell labeling mix (Amersham Pharmacia Biotech) was added to cultures at the time of induction.

Partial purification of σC synthesized in bacteria

About 9 to 13 h postinduction, bacteria were centrifuged at 5000 rpm for 10 min in a JA-20 rotor (Beckman Instruments, Palo Alto, CA). The pellet was washed twice with phosphate-buffered saline (PBS: 0.14 M NaCl, 3 mM KCl, 10 mM phosphate buffer [pH 7.4]) and resuspended in 10 mM Tris–HCl (pH 8.1) at a density of 100 mg bacteria/ml. The suspension was passed through a French press (1000 psi) and then sonicated in a Vibra-Cell (Sonics & Materials, Newtown, CT) for 5 min (2-s pulses, setting 50). The disrupted bacteria were pelleted at 15,000 *g* for 10 min. The pellet was washed in washing solution (10 mM Tris–HCl [pH 8.1], 10 mM EDTA, 0.5% Triton X-100) for 20 min at room temperature (RT). The sample was centrifuged and the pellet (inclusion bodies) was washed twice with PBS. The inclusion bodies were partially solubilized with 2 M urea in 10 mM Tris–HCl (pH 8.1) for 1.5 h, and then centrifuged. Then 8 M urea and 1% β-mercaptoethanol in 10 mM Tris–HCl (pH 8.1) was added to the pellet and the mixture was incubated at RT for 2 h. The sample was centrifuged and the supernatant extensively dialyzed against 10 mM Tris–HCl (pH 8.1). After dialysis, the sample was centrifuged again to remove aggregated material.

Preparation and characterization of polyclonal antibodies to recombinant σC from *E. coli*

The supernatant from the final centrifugation of the σC purification procedure was used to raise polyclonal antibodies in a rabbit. The specificity of the serum obtained for σC was determined by an enzyme-linked immunosorbent assay (ELISA) with bovine serum albumin (BSA) and adenovirus fiber protein expressed and purified in the same way as negative controls. A preimmune serum was also tested in parallel. Polystyrene enzyme immunoassay (EIA) plates (Costar, Cambridge, MA) were coated with purified σC (final supernatant after renaturation), 1% BSA, or purified recombinant adenovirus fiber protein. After an overnight incubation at 4°C, the plates were washed and quenched for 2 h at RT with 5% fat-free milk powder. All the dilutions and washing steps were done in PBS–Tween 20 (0.05%) (Sigma Chemical Co.). Then a twofold dilution series of rabbit polyclonal serum against recombinant σC was added to the plates and incubated for 1 h at RT. After three washes, a 1/4000 dilution of alkaline phosphatase (AP)-conjugated goat anti-rabbit-IgG antibodies (Sigma Chemical Co.) was added to the wells and incubated for 1 h at RT. After extensive washing the substrate for the AP was added and absorbance was measured at 490 nm.

Assessment of monomeric and oligomeric σC concentration

A sample of the partially purified ^{35}S-labeled σC protein from bacteria was incubated for 5 min in Laemmli sample buffer either at 30°C (nondissociating conditions) or at 90°C (dissociating conditions), and then run in 10% SDS–PAGE, to detect oligomeric and monomeric σC protein, respectively, as previously described (Martínez-Costas *et al.,* 1997). As a standard, a twofold dilution series of BSA in PBS was loaded in the same gel. The gel was stained with 0.25% Coomassie brilliant blue in 30% methanol/10% acetic acid, and destained in the same solution without the dye. The bands corresponding to monomeric and oligomeric σC protein were scanned and their concentrations were deduced from the standard curve obtained after scanning the BSA bands. Oligomeric σC protein accounted for 75.3% of the total material when expressed as monomers.

Binding experiments with CEFs

For binding assays, monolayers of 2×10^6 CEFs were washed twice with PBS and incubated with 250 μl of PBS containing 1% BSA for 15 min at 4°C. The solution was removed and cells were then overlaid with 50 μl of a σC-containing sample. PBS (containing 1% BSA) was added to a final volume of 250 μl and the mixture was incubated for 2 h at 4°C with intermittent shaking. After 10 washes with 1 ml of ice-cold PBS, cells were lysed by

adding 30 μl of lysis buffer (1% Nonidet-P40, 0.5% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride) and 100 μl of sterile H_2O, and then incubated for 1 h at 4°C. Nuclei were removed by centrifugation, and the resulting supernatant was lyophylized to reduce the sample volume to 10 μl. Finally, the samples were subjected to SDS–PAGE analysis and protein σC was visualized by either autoradiography or immunoblotting using polyclonal anti-σC antibodies.

For competition experiments, purified recombinant σC protein was added to the monolayers in the presence of BSA and incubated on ice for 90 min. After extensive washing, the monolayers were overlaid with 200 μl of binding buffer containing 4 μg of ^{35}S-labeled reovirus. After 90 min of absorption, the radioactivity of the supernatants and cell fractions was measured as before.

For assays of binding kinetics, approximately 300 ng of ^{35}S-labeled σC in PBS was incubated in the presence of 0.1 mg/ml of BSA with monolayers of 6.5×10^5 CEFs. After different periods of absorption on ice, the monolayers were processed and radioactivity was counted.

For assays of binding saturability, increasing amounts of either ^{35}S-labeled recombinant σC from *E. coli* or ^{35}S-labeled reovirions in PBS containing 0.1 mg/ml of BSA were added to the monolayers. After 90 min of absorption on ice, the monolayers were washed and the radioactivity of both the cell fraction (bound σC) and the supernatant (free σC) was determined.

SDS–PAGE and Western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% polyacrylamide gels was carried out as described by Laemmli (Laemmli, 1970), except that boiling of the samples (dissociating conditions) was replaced in some experiments by incubation at 30°C (nondissociating conditions). After electrophoresis, proteins were fixed in 33% methanol/10% acetic acid for 30 min and autoradiographed by exposure to Agfa Curix AFW X-ray films or transferred to nitrocellulose filters (BioRad, Hercules, CA) for 60 min at 100 mA in a semidry blotting apparatus. Finally, monomeric and oligomeric σC were detected with polyclonal antibodies against reovirions or against protein σC.

Immunoprecipitation

Cultures of pIL-σC bacteria were induced with 3-β-indoleacrylic acid and labeled with ^{35}S cell-labeling mix (Amersham Pharmacia Biotech) for 3 h. The cells were then disrupted and the inclusion bodies were incubated in washing solution. After centrifugation, the supernatant was mixed with one-tenth volume of preimmune serum or anti-σC serum. The samples were incubated overnight at 4°C and then supplemented with two volumes of a protein A–Sepharose suspension (Amersham Pharmacia Biotech) in RIPA buffer (150 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 8.0). After another 45 min of incubation, the samples were centrifuged, the pellet was thoroughly washed with RIPA buffer, and the final pellet was boiled in Laemmli sample buffer (Laemmli, 1970). The resulting mixtures were then analyzed by SDS–PAGE and autoradiography.

In vitro expression of protein σC

The DNA region encoding protein σC was PCR-amplified from plasmid pIL-σC using the primers 5′-GCG TAA TAC GAC TCA CTA TAG GCG GTC TTG TC T TAT AGT TC-3′ (identical to the 5′ end of the third ORF of the S1 gene segment and bearing the T7 RNA polymerase promoter at its 5′ end), and 5′-AAC CAA TCC CAG TAC GGG GC-3′ (complementary to the 3′ end of the same ORF). The PCR products were analyzed on a 1% agarose gel in the presence of EtBr (Sambrook *et al.,* 1989), and the amplified DNA containing the σC ORF was extracted from the gel with phenol:chloroform:isoamyl alcohol (24:24:1), precipitated with 5 M ammonium acetate and isopropanol, and subsequently transcribed with T7 RNA polymerase using the RiboMAX system for RNA production (Promega), using the conditions specified by the manufacturer. The resulting RNA was translated in a rabbit reticulocyte lysate in the presence of ^{35}S-labeled methionine, according to the manufacturer's specifications (Promega). Reactions were incubated at 30°C for 1 h and the products were analyzed by SDS–PAGE.

Expression of protein σC in CEFs

The third ORF of the S1 gene segment, present in the recombinant plasmid pBluescript-S1, was amplified by PCR using the primers 5′-CGG AAT TCT TCA TTG GGA TGG CGG G-3′ and 5′-CCC GTC GAC TTA GGT GTC GAT GCC GGT AC-3′, and subsequently cloned into the *Eco*RI and *Sal*I sites of the expression vector pCI-neo (Promega). Transfection of CEF cells was performed using the LipofectAMINE PLUS reagent (Gibco BRL) following the indications of the manufacterer. Before adding the transfection reagents, the pCIneo-σC DNA was mixed with the translation-enhancing vector pAdVAntage (Promega) at a 10:1 ratio. Negative controls lacking one of the recombinant plasmids or lacking the lipofectamin reagent were also included. The transfected cells were incubated at 37°C overnight, collected in lysis buffer (10 mM HEPES, 1 mM DTT, 50 mM NaCl, 0.5% Nonidet P-40, 10% glycerol), and incubated on ice for 15 min. Nuclei were pelleted by low-speed centrifugation and the resulting supernatant was used for oligomerization and cell-binding studies.

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