Possible Involvement of the Double-Stranded RNA-Binding Core Protein σ A in the Resistance of Avian Reovirus to Interferon

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Treatment of primary cultures of chicken embryo fibroblasts with a recombinant chicken alpha/beta interferon (rcIFN) induces an antiviral state that causes a strong inhibition of vaccinia virus and vesicular stomatitis virus replication but has no effect on avian reovirus S1133 replication. The fact that avian reovirus polypeptides are synthesized normally in rcIFN-treated cells prompted us to investigate whether this virus expresses factors that interfere with the activation and/or the activity of the IFN-induced, double-stranded RNA (dsRNA)-dependent enzymes. Our results demonstrate that extracts of avian-reovirus-infected cells, but not those of uninfected cells, are able to relieve the translation-inhibitory activity of dsRNA in reticulocyte lysates, by blocking the activation of the dsRNA-dependent enzymes. In addition, our results show that protein σ A, an S1133 core polypeptide, binds to dsRNA in an irreversible manner and that clearing this protein from extracts of infected cells abolishes their protranslational capacity. Taken together, our results raise the interesting possibility that protein σ A antagonizes the IFN-induced cellular response against avian reovirus by blocking the intracellular activation of enzyme pathways dependent on dsRNA, as has been suggested for several other viral dsRNA-binding proteins.

The alpha/beta interferons (IFNs) are a family of multifunctional cytokines encoded by intronless genes, which are expressed and secreted by leukocytes and fibroblasts in response to viral infection and which have the same cell receptors (for recent reviews, see references 15, 22, 42, and 53). Extracellular IFNs bind to specific high-affinity cell surface receptors to trigger the activation of signal transduction pathways that, through a phosphorylation cascade, induce increased expression of the designated IFN-responsive genes (for reviews, see references 19, 43, 57, and 63). Three of the many alpha/beta IFN-inducible gene products have been shown to play an important role in fighting virus infection, namely, Mx proteins, the 2',5'-oligoadenylate synthetase system (2-5A synthetase), and the double-stranded RNA (dsRNA)-activated protein kinase (PKR) (for reviews, see references 19, 28, 32, 39, 41, 45, 56, and 57). Mx proteins are a family of related GTPases that are thought to inhibit the viral polymerase activity of susceptible viruses (reviewed in reference 40). Both 2-5A synthetase and PKR antiviral pathways play a key role in the intracellular regulation of protein synthesis. Increased expression of these enzymes is induced by IFN, but they are latent until after activation by dsRNA (28, 45). The activated 2-5A synthetase catalyzes the synthesis of short oligonucleotides of the general structure ppp(A2'p5')nA. These oligonucleotides bind and stimulate a latent endoribonuclease, designated RNase L, to degrade both cellular and viral RNAs, thus preventing protein synthesis (for reviews, see references 44 and 54). Interaction of PKR with dsRNA results in autophosphorylation and dimer-

ization of the protein, and the active enzyme catalyzes serine/threonine phosphorylation of different proteins, including the alpha subunit of protein synthesis eukaryotic initiation factor 2 (for reviews, see references 8, 9, 46, 47, and 59). Phosphorylation of the alpha subunit of eukaryotic initiation factor 2 results in inhibition of protein synthesis at the initiation step of translation (10). In order to sustain a productive infection, many viruses utilize strategies to counteract the antiviral action of IFNs. The best characterized of these countermechanisms are those that block the function of PKR (for recent reviews, see references 21 and 32). Virus-induced inhibition of RNase L has also been reported previously (4, 5).

Avian reoviruses are members of the *Orthoreovirus* genus, one of the six genera of the *Reoviridae* family (30). They are nonenveloped viruses that replicate in the cytoplasm of infected cells and that contain 10 dsRNA genome segments enclosed within a double protein capsid shell of 70 to 80 nm in diameter (55). In spite of the importance of avian reoviruses as avian pathogens that cause important losses in poultry farming, very little is known about the basic aspects of their biology. Our laboratories have been working for several years on the molecular biology of the avian reoviruses and on the molecular mechanisms that regulate their interactions with the host cell. The recent availability of a recombinant chicken alpha/beta interferon (rcIFN) (50) prompted us to investigate its effect on the replication of avian reovirus S1133 in primary cultures of chicken embryo fibroblasts (CEF). A previous study revealed that four avian reovirus strains, including S1133, were resistant to the antiviral action of a natural chicken IFN produced in embryonated eggs (16). The results presented here demonstrate that exposure of CEF to rcIFN induces a strong intracellular antiviral state that inhibits the replication of vesicular stomatitis virus (VSV) and vaccinia virus but not the replica-

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tion of avian reovirus S1133. We also found that the avian reovirus core polypeptide σA is a dsRNA-binding protein that is able to abolish the capacity of dsRNA to inhibit translation in reticulocyte lysates.

MATERIALS AND METHODS

Cells and viruses. Primary cultures of CEF were prepared from 9- to 10-day-old chicken embryos as described previously (55). Cells were incubated in medium 199 supplemented with 10% tryptose phosphate broth and 5% calf serum. Strain S1133 of avian reovirus (61) was grown on semiconfluent monolayers of CEF. Conditions for growing, purifying, and determining the titer of the virus have been described previously (55). Propagation of vaccinia virus and VSV was essentially as described previously (13, 24).

Infections and preparation of cytoplasmic extracts. Semiconfluent CEF monolayers were incubated with 10 PFU of the indicated virus per cell for 2 h at 37°C. Then, unadsorbed virus was removed (this moment was considered time zero of infection), and cells were overlaid with medium 199 containing 2.5% fetal calf serum and incubated at 37°C. Metabolic radiolabeling of proteins was performed by incubating the cell monolayers in methionine-free medium containing 2.5% dialyzed fetal calf serum and supplemented with 100 μ Ci of [35 S]methionine per ml for 1 h at 37°C. Cells were lysed at a concentration of 3×10^7 cells/ml in lysis buffer (10 mM Tris-HCl [pH 8.0], containing 10 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100), and nuclei were removed by low-speed centrifugation. The resulting supernatant was designated S10 extract. Particulate material was removed from S10 extracts by centrifugation in a Beckman SW50.1 rotor at 40,000 rpm for 2 h. The recovered supernatant was designated S100 extract.

Phosphorylation of dsRNA-binding proteins. S10 extracts were incubated for 10 min at 4°C with an equal volume of poly(I-C)-agarose beads (Amersham Pharmacia Biotech) in lysis buffer. After extensive washing of the beads with the same buffer, the affinity matrix was incubated for 30 min at 37°C with 50 μ Ci of [γ - 32 P]ATP per ml, then washed with 50 volumes of lysis buffer, and boiled in Laemmli sample buffer (31). After centrifugation, supernatant proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography.

2-5A synthetase assay. S100 extracts were incubated with equal volumes of poly(I-C)-agarose beads in binding buffer (10 mM HEPES [pH 7.6], containing 50 mM KCl, 1.5 mM magnesium acetate, 7 mM 2-mercaptoethanol, and 20% glycerol) for 15 min at 30°C. The beads were washed extensively with the same buffer and then incubated for 10 h at 30°C with 250 μ Ci of [2,5,8- 3 H]ATP per ml. After centrifugation, 7.5 μ l of the supernatants was incubated for 2 h at 37°C with 10 U of bacterial alkaline phosphatase per ml. The mixture was spotted onto DEAE filter discs (Whatman), and the discs were then washed with distilled water, dried, and subjected to scintillation counting.

In vitro translation. In vitro translation assays were performed with micrococcal nuclease-treated rabbit reticulocyte lysates (Promega) programmed with 13 μ g of exogenous tobacco mosaic virus (TMV) RNA (Roche Molecular Biochemicals) per ml as recommended by the manufacturer. Incubations were allowed to proceed for 1 h at 30°C, and then samples were boiled in Laemmli sample buffer (31) and analyzed by SDS-PAGE and autoradiography.

Isolation and labeling of S1133 dsRNA and gel shift assays. Genomic dsRNA was isolated from purified S1133 reovirions by phenol extraction. After ethanol precipitation, the RNA pellet was dissolved in water and passed through a MicroSpin TM-400-HR column (Amersham Pharmacia Biotech) to remove small oligonucleotides. For radiolabeling, 10 μ g of dsRNA was incubated for 16 h at 4°C, with 8 U of T4 RNA ligase in 20 μ l of reaction buffer (50 mM Tris-HCl [pH 8.0], containing 10% dimethyl sulfoxide, 4 mM MgCl₂, 50 μ M ATP, 1 mM dithiothreitol, 10 μ g of bovine serum albumin per ml, and 15 mCi of [32 P]pCp per ml). Excess pCp was removed by passing the samples through MicroSpin TM-S200-HR columns (Amersham Pharmacia Biotech), and the flowthrough fraction was phenol extracted and ethanol precipitated. For the gel shift assays, samples containing 10,000 cpm of radiolabeled dsRNA were incubated at room temperature for 15 min with 10 μ l of S100 extract and then separated by nondenaturing PAGE (14).

Electrophoretic analysis. SDS-PAGE was performed as described by Laemmli (31). For nondenaturing PAGE, SDS and 2-mercaptoethanol were removed from all solutions. After electrophoresis, gels were fixed in an aqueous solution containing 33% methanol and 10% acetic acid and then dried and exposed to X-ray film (Agfa-Curix AFW).

Affinity chromatography. Purified S1133 dsRNA was coupled to activated Sepharose 4B (Amersham Pharmacia Biotech) following the instructions of the manufacturer. Fifty microliters of dsRNA-Sepharose beads was incubated for 15 min at room temperature with an equal volume of S100 extract, in lysis buffer supplemented with 150 mM NaCl. After five washes with 1 ml of the same buffer, the beads were washed successively with 1 ml of the same buffer containing first 1 M and then 2 M NaCl. After the final wash, the beads were boiled in Laemmli loading buffer (31) and centrifuged. Supernatants from the different washes and from the final centrifugation were subjected to SDS-PAGE and autoradiography.

Immunodepletion and immunoprecipitation. Nonradiolabeled S100 extracts were incubated for 12 h at 4°C with reovirus-specific anti-S1133 polyclonal

FIG. 1. Comparative analysis of the intracellular levels of PKR and 2-5A synthetase in CEF treated with different concentrations of rIFN. CEF monolayers were incubated with the amounts of rIFN indicated, and 20 h later, cells were lysed and S10 and S100 extracts were prepared as described in Materials and Methods. (A) Poly(I-C)-agarose-retained proteins from S10 extracts were incubated in the presence of [γ - 32 P]ATP and subsequently analyzed by SDS-PAGE and autoradiography. (B) S100 extracts were incubated with poly(I-C)-agarose beads in the presence of [2,5,8- 3 H]ATP for 10 h at 30°C. After centrifugation, the resulting supernatants were digested with bacterial alkaline phosphatase, and samples were spotted onto DEAE filter discs. Filters were washed with water, dried, and subjected to scintillation counting. B, buffer only.

antibodies or with an anti- σA monoclonal antibody; an equal volume of protein A-Sepharose was then added, the resulting mixture was incubated for 30 min at 4°C and then centrifuged, and the resulting supernatant was considered the σA -depleted extract. The efficacy of this procedure for depletion of σA was confirmed by immunoprecipitation. [35 S]methionine-labeled extracts were processed in the same way, and after centrifugation, the resulting pellets were exhaustively washed with cell lysis buffer and boiled in Laemmli sample buffer. Radioactive proteins were then resolved by SDS-PAGE and visualized by autoradiography (see Fig. 7C). The preparation and characterization of the anti- σA monoclonal antibody 42-9 have been described elsewhere (60).

RESULTS

rcIFN induces a strong antiviral state in primary cultures of CEF. The IFN used in this study has been shown to be a powerful inducer of the Mx gene promoter and also to inhibit VSV replication in CEC32 cells (50, 51). To further characterize the antiviral properties of this rcIFN, we first investigated its capacity to induce increased expression of PKR and 2-5A synthetase in primary cultures of CEF. To this end, CEF monolayers were treated with different doses of rcIFN for 20 h, then cells were lysed, and S10 and S100 cell extracts were prepared. Intracellular levels of PKR were monitored by PKR autophosphorylation in S10 extracts incubated with poly(I-C)-agarose in the presence of [γ - 32 P]ATP. The autoradiogram shown in Fig. 1A revealed the presence of a 70-kDa phosphorylated polypeptide band in extracts of IFN-treated cells but not in extracts of control cells. The intensity of the radiolabeled band increased with IFN dose. In the absence of any information about the nucleotide sequence of the chicken PKR mRNA which would allow analysis of intracellular levels of the PKR mRNA, we decided to use antibodies raised against mammalian PKRs in an attempt to measure intracellular levels of the chicken PKR. Unfortunately, these antibodies did not recognize the avian enzyme in immunoprecipitation assays or in Western blots. In spite of this, we consider it very likely that the 70-kDa protein detected in extracts of IFN-treated cells is the chicken PKR, because of its molecular mass and also because it binds to dsRNA, is induced by IFN, and is phosphorylated. Intracellular levels of 2-5A synthetase were determined by measuring the capacity of the S100 extracts to synthesize bacterial alkaline phosphatase-resistant oligonucleotides upon incubation with poly(I-C)-agarose beads in the presence of [2,5,8- 3 H]ATP. As can be seen in Fig. 1B, the amount of phosphatase-resistant radiolabeled material retained on DEAE filter discs increased with the dose of rcIFN to which

FIG. 2. Effect of rcIFN on protein synthesis and on viral replication in CEF infected with avian reovirus S1133, vaccinia virus, or VSV. CEF monolayers were incubated with the indicated IFN doses for 20 h and then infected with the corresponding virus. (A) At 20 h postinfection, cells were incubated for 1 h with [³⁵S]methionine and then lysed, and the resulting cytoplasmic extracts were subsequently analyzed by SDS-PAGE and autoradiography. (B) At 20 h postinfection, infected cells were collected, subjected to three cycles of freeze-thawing, and then centrifuged. The concentration of infectious viral particles in the supernatants was determined by plaque assay on fresh CEF monolayers. ▲, S1133; ■, vaccinia virus; ●, VSV.

the cells were exposed. Taken together, our results indicate that rcIFN induces increased expression of PKR and 2-5A synthetase in CEF. Similar results were obtained when the intracellular levels of these enzymes were monitored 30 and 40 h after the addition of IFN. These findings, combined with the previously reported induction by rcIFN of the Mx promoter, clearly demonstrate that the rcIFN used in this study is able to induce a strong antiviral state in chicken cells.

Avian reovirus S1133, but not vaccinia virus or VSV, is resistant to the antiviral action of rcIFN. To determine the sensitivity of avian reovirus S1133 to rcIFN and to directly assess the antiviral activity of this IFN, protein synthesis and the production of infectious particles were investigated in IFN-treated CEF infected with avian reovirus S1133, vaccinia virus, and VSV. Vaccinia virus and VSV were chosen as control viruses because they replicate efficiently in CEF and also because their sensitivity to avian IFN has been well documented. Specifically, VSV replication in CEF is very sensitive to chicken IFN, and therefore this virus is currently used to measure the antiviral activity of IFN preparations (37, 50–52). Vaccinia virus replication in CEF is inhibited by chicken IFN by posttranscriptional mechanisms (17, 23, 24). The results of our experiments, shown in Fig. 2, reveal that both protein synthesis (Fig. 2A) and the production of infectious particles (Fig. 2B) were severely reduced in IFN-treated CEF infected with vaccinia virus and VSV, and they also showed that VSV was more sensitive to rcIFN than vaccinia virus. In marked contrast, rcIFN had no effect on protein synthesis in either uninfected or S1133-infected cells (Fig. 2A). Furthermore, the production of infectious virus particles in S1133-infected CEF was not significantly affected by the IFN treatment, even when IFN doses as high as 1,000 U/ml were used (Fig. 2B). Overall, our results demonstrate that the induction of an antiviral state by rcIFN in CEF is not sufficient to inhibit the replication of avian reovirus S1133.

Effect of extracts of S1133-infected cells on the capacity of dsRNA to block in vitro translation. The fact that avian reovirus protein synthesis takes place normally in rcIFN-treated CEF suggests that the endogenous PKR and 2-5A synthetase

are not functionally active in the IFN-treated infected cells. This would occur if an inducer of these enzymes is not produced during the viral infection or if the activation and/or the activity of these enzymes is inhibited by factors present in the avian reovirus-infected cell. Since there is compelling evidence that dsRNA inhibits translation in reticulocyte lysates by causing activation of endogenous PKR and 2-5A synthetase (27), we next investigated whether extracts of infected cells were able to restore the translation capacity of a dsRNA-treated reticulocyte lysate. First, the translation-inhibitory activity of avian reovirus S1133 dsRNA in our reticulocyte lysate was titrated (Fig. 3A). Translation of exogenous TMV mRNA was completely blocked in the presence of 10 ng of dsRNA per ml but was partially restored when the dsRNA concentration was increased to 100 and 1,000 ng/ml, as has been previously reported (27). Unless otherwise indicated, a final dsRNA concentration of 10 ng/ml was used in our standard in vitro translation experiments. The translation-inhibitory activity of viral dsRNA remained intact after preincubation with an S100 extract from uninfected CEF (Fig. 3B, lanes U), but not after preincubation with an S100 extract from S1133-infected cells (Fig. 3B, lanes I). The capability of the extract from infected cells to block the translation-inhibitory activity of dsRNA was dose dependent (Fig. 3C). These results indicate that a factor present in S1133-infected cells is able to block the inhibition of translation induced by dsRNA in reticulocyte lysates.

Mechanism by which extracts of infected cells allow in vitro translation to occur in the presence of dsRNA. Extracts of infected cells might block the translation-inhibitory activity of dsRNA in reticulocyte lysates by any of several mechanisms. The possibilities are (i) by blocking the activation of IFN-inducible enzymes, (ii) by inhibiting the activity of these enzymes, and (iii) by acting as a supplementary source of dsRNA, thus increasing the dsRNA concentration to a noninhibitory level. In order to discriminate among these possibilities, additional in vitro translation experiments were performed. First, a fixed amount of an S100 extract from infected cells was incubated with increasing amounts of dsRNA. As shown in Fig. 4A, increasing the dsRNA concentration caused a reduction in the

FIG. 3. Translation of TMV mRNA in reticulocyte lysates supplemented with dsRNA and CEF S100 extracts. (A) S1133 dsRNAs, at the indicated final concentrations, were incubated with reticulocyte lysates for 10 min at 30°C, and then the mixtures were supplemented with TMV mRNA (final concentration, 13 µg/ml) and [³⁵S]methionine. After 1 h at 30°C, samples were analyzed by electrophoresis and autoradiography. (B) Cytoplasmic S100 extracts (1 µl) from both uninfected CEF (U) and avian reovirus-infected CEF (I) were added to reticulocyte lysates and incubated with (lanes +) or without (lanes -) viral dsRNA for 10 min at 30°C. Then the mixtures were supplemented with 13 µg of TMV mRNA per ml and incubated for 1 h at 30°C. Translation mixtures were analyzed as described for panel A. The conditions for lane I+ of Fig. 3B will be designated hereafter as the standard *in vitro* translation. (C) A standard *in vitro* translation was performed, except that dsRNA was incubated with 1 µl of 0, 1/2, 1/3, 1/5, and 1/10 dilutions (from right to left) of the S100 extract from infected cells.

capacity of the extract to rescue *in vitro* translation. This result rules out the possibility that the observed effect of the S100 extract is attributable to dsRNA supplementation and also suggests that the S100 extract blocks the activation rather than the activity of the dsRNA-dependent enzymes. To confirm this possibility, the order of addition of both dsRNA and S100 extract to reticulocyte lysates was changed (Fig. 4B). Compared with the standard conditions (lane -10), inhibition of the translation capacity of the reticulocyte lysate was observed both when the dsRNA, the S100 extract, and the mRNA were

FIG. 4. Effects on *in vitro* translation of dsRNA concentration and of the order of addition of dsRNA and S100 extract. (A) A standard *in vitro* translation was carried out, except that a fixed amount of an S100 extract from infected cells was incubated with the indicated concentrations of dsRNA. (B) Lane -10, standard *in vitro* translation; lane 0, S100 extract, dsRNA, and TMV mRNA were all added together to the reticulocyte lysate without prior preincubation; lane +5, dsRNA was incubated with the reticulocyte lysate for 5 min, then the mixture was supplemented with the S100 extract, and the incubation proceeded for another 5 min. Finally, TMV mRNA was added to the final mixture, and translation proceeded at 30°C for 1 h.

FIG. 5. Mobility shift assays. (A) Increasing amounts of S100 extracts from either uninfected CEF (U) or avian reovirus-infected CEF (I) were incubated with a fixed amount of ³²P-labeled viral dsRNA at room temperature for 15 min. Mixtures were then analyzed by electrophoresis on nondenaturing polyacrylamide gels, and radioactive bands were visualized by autoradiography (the electrophoretic positions of the L, M, and S classes of S1133 genomic segments are indicated at the left of the figure). (B) An experiment similar to that shown in panel A, but S100 extracts from infected cells were preincubated with the indicated nonradioactive nucleic acids, before addition of the radiolabeled viral dsRNAs. ss, single stranded.

added together (lane 0) and when first the dsRNA, then the S100 extract, and finally the mRNA were added (lane +5). These results clearly support the idea that the S100 extract from infected cells is blocking the activation of the dsRNA-dependent enzymes rather than inhibiting their functional activities.

Cytoplasmic extracts of infected cells contain a dsRNA-binding protein. Several viruses have been shown to code for proteins that specifically bind to dsRNA. Some of these proteins are believed to play a key role in counteracting the antiviral action of IFNs (32, 50, 63). To determine whether a similar dsRNA-binding activity was present in avian reovirus-infected cells, a fixed amount of ³²P-labeled S1133 dsRNAs was incubated with increasing amounts of S100 extracts from mock-infected or avian reovirus-infected CEF, and the resulting mixtures were analyzed by native PAGE and autoradiography (Fig. 5). The results show that all 10 viral dsRNA segments were shifted to a complex migrating as a single shifted band upon incubation with an extract from infected cells (Fig. 5A, lanes I) but not upon incubation with an extract from uninfected cells (Fig. 5A, lanes U). The binding activity was specific for dsRNA, since the dsRNA shifting was inhibited only by dsRNA, and not by single-stranded RNA, single-stranded DNA, or double-stranded DNA (Fig. 5B). We also found that the dsRNA-binding activity was protease sensitive

FIG. 6. Identification of the dsRNA-binding polypeptide present in extracts of infected cells. (A and B) S100 extracts (lanes E) from S1133-infected cells (A) or from uninfected cells (B) were incubated with a resin consisting of genomic S1133 dsRNAs covalently attached to Sepharose, as described in Materials and Methods. The resins were washed successively with buffer containing 0.15 M (lanes 1), 1 M (lanes 2), and 2 M (lanes 3) NaCl. Then the resin beads were boiled in Laemmli sample buffer and centrifuged. The original extracts, the washes, and the final supernatants (lanes SB) were analyzed by SDS-PAGE and autoradiography. The positions of the three size classes of viral polypeptides are shown on the right of panel A. (C) ^{35}S -labeled bands corresponding to avian reovirion polypeptides σA (σA -reovirion) and λC (λC -reovirion) and to the polypeptide retained on dsRNA-Sepharose (dsRNA-retained) were excised from SDS-polyacrylamide gels and digested with the indicated amounts of V8 protease, as previously described by Cleveland (11). Digested products were analyzed by SDS-PAGE and fluorography.

but not nuclease sensitive (results not shown). These results demonstrate that a dsRNA-binding protein is present in S1133-infected CEF.

Identification of the dsRNA-binding protein present in infected cells. To identify the dsRNA-binding protein present in infected cells, [^{35}S]methionine-labeled S100 extracts from either uninfected or S1133-infected cells were incubated with a resin consisting of viral dsRNAs covalently attached to Sepharose. After several washings, the Sepharose beads were boiled in Laemmli sample buffer and centrifuged, and supernatants and washes were analyzed by SDS-PAGE. The autoradiograms shown in Fig. 6 indicate that a dsRNA-binding polypeptide is present in extracts from infected cells (Fig. 6A, lane SB) but not in extracts from uninfected cells (Fig. 6B). The finding that the dsRNA-binding polypeptide remained attached to the matrix after the dsRNA-Sepharose beads were washed with a 2 M NaCl-containing buffer revealed that this polypeptide possesses a strong dsRNA-binding affinity (Fig. 6A, lane 3). This polypeptide had the same electrophoretic mobility as the viral protein σA , and therefore it could be either avian reovirus protein σA or a similar-size cell-encoded polypeptide whose synthesis is induced during viral infection. To ascertain the identity of the dsRNA-retained polypeptide, V8 peptide mapping of both the dsRNA-retained polypeptide and authentic reovirion σA was performed (11). As can be seen in Fig. 6C, both polypeptides, but not avian reovirion λC , yielded identical

peptide maps, confirming that the dsRNA-retained polypeptide is avian reovirus protein σA . This is the first time that a dsRNA-binding activity has been reported for avian reovirus.

Protein σA is the dsRNA translation-inhibition blocker present in extracts of infected cells. Having demonstrated that protein σA is the major dsRNA-binding protein present in infected cells, we next investigated whether this protein blocks the capability of dsRNA to inhibit in vitro translation. Since purified σA protein was not available to perform a direct assay of its activity, we followed an indirect approach of comparing the translational rescuing efficiency of an S100 extract before and after removal of σA protein. The extract was depleted of protein σA by either incubation with dsRNA-Sepharose (Fig. 7A) or immunodepletion (Fig. 7B), since both treatments were found to be effective in removing protein σA from the extract (Fig. 6A, lane SB; Fig. 7C, lane 5). As shown in Fig. 7C, preimmune serum (lane 3) did not recognize any of the proteins in the extract (lane 2), whereas anti-S1133 antibodies recognized most of the viral structural polypeptides (lane 4), and the anti- σA monoclonal antibody immunoprecipitated protein σA specifically (lane 5). Preincubation of the extract of infected cells with either Sepharose (Fig. 7A, lane 1) or preimmune serum (Fig. 7B, lane 2) did not affect its protranslational activity. However, this activity was lost after preincubation of the extract with dsRNA-Sepharose beads (Fig. 7A, lane 2) or after immunodepletion with either a monoclonal anti- σA antibody (Fig. 7B, lane 3) or polyclonal antireovirion antibodies (Fig. 7B, lane 4). Taken together, these results demonstrate that σA is the protranslational factor present in extracts of S1133-infected cells. Our results also suggest that this biological activity of σA in reticulocyte lysates is linked to its ability to bind and sequester activator dsRNA from the dsRNA-dependent enzymes.

DISCUSSION

Treatment of cultured cells with IFN inhibits the replication of many viruses, but for most virus-cell systems, the molecular mechanisms responsible for this inhibition remain largely unknown. The results shown in Fig. 1 and 2 of the present work, and those previously reported by other laboratories (50, 51), clearly demonstrate that the rIFN used in this study is a powerful antiviral-state inducer in chicken cells. The inhibition of viral protein synthesis observed in rIFN-treated cells infected with VSV and vaccinia virus suggests that replication of these viruses is being affected at a translational or a pretranslational step. In the case of vaccinia virus, inhibition of early viral protein synthesis and degradation of early viral mRNAs have been shown to occur in IFN-treated CEF (13, 17, 24), suggesting that the activities of both PKR and 2-5A synthetase play an important role in the IFN-sensitive phenotype of this virus in chicken cells. In most cell lines tested, vaccinia virus has been reported to be resistant to IFN (66), and this resistance has been associated with the activity of two "anti-antiviral" proteins encoded by the virus. Thus, the vaccinia virus *E3L* gene encodes a dsRNA-binding protein with strong affinity for dsRNA and has been shown to inhibit the activation of PKR and to suppress the 2-5A synthetase pathway in several infected cell lines (2, 7). In addition, vaccinia virus also encodes the K3L gene product that is believed to inhibit PKR by acting as pseudosubstrate (65). However, our finding that treatment of CEF with rIFN causes a drastic inhibition of vaccinia virus replication in chicken cells raises the possibility that mechanisms other than the 2-5A synthetase and PKR pathways contribute to the antiviral effects of IFN, as previously suggested by several authors (18, 34). Such "other" mechanisms are likely

FIG. 7. Activity of the extracts from infected cells after removal of protein σ A by dsRNA-Sepharose chromatography or immunodepletion. (A and B) Electrophoretic analysis of the products of standard in vitro translation reactions in which dsRNA was incubated with the supernatants resulting from the centrifugation of infected cell extracts that had been preincubated with Sepharose (panel A, lane 1) or dsRNA-Sepharose (panel A, lane 2), or with buffer only (panel B, lane 1), preimmune serum (panel B, lane 2), a specific anti- σ A monoclonal antibody (panel B, lane 3), or polyclonal anti-S1133 antibodies (panel B, lane 4). (C) A [35 S]methionine-radiolabeled S100 extract of avian reovirus-infected cells (lane 2) was subjected to immunoprecipitation with preimmune serum (lane 3), anti-S1133 polyclonal antibodies (lane 4), or an anti- σ A monoclonal antibody (lane 5). Lane 1, electrophoretic analysis of purified S1133 reovirions.

to be responsible for the drastic inhibition of VSV replication that we observed in IFN-treated CEF, since it has been demonstrated that pretreatment of primary CEF with chicken IFN reduces primary transcription of VSV by 60 to 70% (38). However, the fact that replication of vaccinia virus in IFN-treated CEF is inhibited by posttranscriptional mechanisms raises the possibility that the activity of these anti-antiviral proteins might not always be sufficient to achieve a complete inhibition of the dsRNA-dependent enzymes. One possible explanation is that different amounts of dsRNA are produced intracellularly when a virus infects different cells. The possibility also exists that major differences in the expression of the anti-antiviral proteins and/or the dsRNA-dependent enzymes occur in different IFN-treated virus-infected cells. In this regard, a remarkable IFN-dependent 10^4 -fold induction of 2-5A synthetase in CEF has been reported (1). It would be of great interest to compare the intracellular activities of the dsRNA-dependent enzymes among different IFN-treated vaccinia virus-infected cells in which the virus shows different IFN sensitivity.

Surprisingly, the antiviral state induced by rcIFN in CEF has no inhibitory effect on avian reovirus replication, a situation similar to that reported for the replication of mammalian reovirus type 3 in either HeLa cells or mouse SC1 fibroblasts (12, 18). Thus, it is clear that the IFN-mediated induction of an intracellular antiviral state is not always sufficient to inhibit virus replication. Our finding that protein synthesis takes place normally in IFN-treated CEF infected with avian reovirus S1133 suggests that neither PKR nor 2-5A synthetase is functionally active in these cells. The inactivity of these enzymes could be due to the absence of an intracellular inducer or to the capacity of the virus to inhibit their activation and/or their activity. The first possibility is very unlikely, since dsRNA is thought to be produced in most viral infections (8), and since our preliminary results suggest that, as happens with mammalian reoviruses (3), the avian reovirus s1 mRNA is a potent activator of PKR (L. Labrada and J. Benavente, unpublished results). In favor of the second possibility is our present finding that extracts from avian-reovirus-infected cells, but not extracts from uninfected cells, are able to block the inhibition of translation induced by dsRNA in reticulocyte lysates. Since there is compelling evidence that dsRNA causes inhibition of transla-

tion in reticulocyte lysates by inducing the activation of endogenous PKR and 2-5A synthetase (27), our results indicate that a factor present in extracts from infected cells down-regulates the activity of these enzymes. Indirect evidence indicates that this factor is the avian reovirus core polypeptide σ A, a dsRNA-binding protein. The results of our in vitro translation experiments further revealed that σ A exerts its protranslational activity by preventing the activation of the dsRNA-dependent enzymes rather than by inhibiting their activities. The fact that binding of σ A to the 10 species of viral dsRNA results in a complex migrating as a single shifted band suggests both that binding of σ A to dsRNA does not cause degradation of the nucleic acid and that the affinity of σ A for dsRNA is not sequence specific. The latter suggestion was further confirmed by the finding that protein σ A also binds to poly(I-C)-agarose (unpublished data). Since this is the first time that an avian reovirus dsRNA-binding protein has been reported, σ A should be included in the growing list of dsRNA-binding proteins of viral origin that interfere with the activation of the IFN-inducible and dsRNA-dependent enzymes, including vaccinia virus E3L (7, 62), influenza virus NS1 (36), mammalian reovirus σ 3 (35, 67), and porcine group C rotavirus NSP3 (33). All these proteins have been reported to confer IFN resistance, by sequestering intracellular dsRNA activators. In an effort to correlate the dsRNA-binding activity of protein σ A with its capacity to block the IFN response in CEF, we looked for avian reovirus strains displaying different sensitivities to rcIFN. Unfortunately, all of the available strains (S1133, 1733, and 2408) are IFN resistant, ruling out the use of recombinant genetics to explore the role of the σ A-encoding gene in determining resistance to IFN.

We have been unable to find consensus dsRNA-binding motifs (6, 29) in the amino acid sequence of protein σ A (unpublished data), as has also been reported for the dsRNA-binding proteins NS1 of influenza virus (25) and VP6 of blue-tongue virus (58). Nevertheless, σ A has a strong affinity for dsRNA, as demonstrated by the finding that it does not detach from dsRNA-affinity resins when the salt concentration of the elution buffer is increased. A similar situation has been reported for other dsRNA-binding proteins including PKR (48) and vaccinia virus protein E3L (26). In contrast, mammalian

reovirus $\sigma 3$ (67) and porcine group C rotavirus NSP3 (33) show a weaker affinity for dsRNA, since they can be eluted from dsRNA-affinity resins by increasing the salt concentration of the elution buffer. It has been suggested that the high intracellular levels of $\sigma 3$ compensate for its low dsRNA affinity in preventing the activation of the dsRNA-dependent enzymes (67). Conversely, a strong affinity for dsRNA would be required for avian reovirus σA to block the intracellular activation of these enzymes, since this protein is present in low copy numbers in infected cells (49).

To further characterize the properties and functional activities of the avian reovirus σA protein, we have recently cloned the σA -encoding gene in different prokaryotic and eukaryotic expression vectors. We are currently experimenting with these constructs with the aim of establishing a causal link between the dsRNA-binding activity of σA and the resistance of avian reovirus to IFN. It would also be interesting to compare the rcIFN sensitivity in CEF of wild-type vaccinia virus with that of a recombinant vaccinia virus that expresses the avian reovirus σA protein.

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