

In Vivo and In Vitro Models of Demyelinating Disease: Activation of the Adenylate Cyclase System Influences JHM Virus Expression in Explanted Rat Oligodendrocytes

S. BEUSHAUSEN,^{1†} S. NARINDRASORASAK,² B. D. SANWAL,² AND S. DALES^{1*}

Cytobiology Group, Department of Microbiology and Immunology,¹ and Department of Biochemistry,² University of Western Ontario, London, Ontario, Canada N6A 5C1

Received 26 May 1987/Accepted 25 August 1987

The specificity of JHM virus (JHMV) tropism for rat oligodendrocytes, as one of the primary host cells in the central nervous system, is maintained after explantation (S. Beushausen and S. Dales, *Virology* 141:89-101, 1985). The temporal correlation between onset of resistance to JHMV infection in vivo, completion of myelination, and maturation of the central nervous system can be simulated in vitro by inducers of oligodendrocyte differentiation (Beushausen and Dales, *Virology*, 1985). Stimulation of differentiation through the elevation of intracellular cyclic AMP (cAMP) levels suggests a possible connection between activation of the adenylate cyclase system and coronavirus expression. Chromatographic analysis of cAMP-dependent protein kinase activity in cytosol extracts prepared from astrocytes or oligodendrocytes revealed that both glial cell types were deficient in protein kinase I, indicating that expression of coronavirus in differentiated cells was not contingent upon the presence of protein kinase I. However, treatment with N⁶,2'-*O*-dibutyryl adenosine-3',5'-cyclic monophosphate (dbcAMP) resulted in a severalfold enhancement of the free regulatory subunit (RI) in oligodendrocytes but not in astrocytes. The RII subunit in both neural cell types was relatively unaffected. Rapid increase in RI due to dbcAMP treatment was correlated with inhibition of JHMV expression. Other differentiation inducers, including 8-Br cAMP and forskolin which, by contrast, caused a decrease in detectable RI, also blocked JHMV expression. This apparent anomaly can be attributed to an increased turnover of RI due to destabilization of the molecule which occurs upon site-specific binding of the cyclic nucleotides. On the basis of these observations, we conclude that the state of oligodendrocyte differentiation manifested with the modulation of RI regulates JHMV expression. The differentiation process did not affect either virus adsorption or sequestration but appeared to inhibit the expression of viral RNA and proteins, implying that replication was inhibited at some step between penetration and initiation of genomic functions, perhaps at the stage of uncoating. We therefore examined the possibility that protein kinases and phosphatases, which influence cellular regulation during cAMP-induced differentiation, may be responsible for the phenomenon of coronavirus suppression in oligodendrocytes. Evidence was obtained indicating that normal processing of the phosphorylated nucleocapsid protein is inhibited in differentiated oligodendrocytes, consistent with the notion that JHMV replication might be arrested during uncoating.

Viruses that infect the central nervous system (CNS) are notorious for their ability to persist or to become latent. Persistent infections are characterized by progressive, chronic disease symptoms and a slow spread of virus. RNA viruses have been associated with chronic demyelinating diseases in humans and other mammals. For example, the persistent nature of measles virus in subacute sclerosing panencephalitis has cast suspicion on viruses as being the causative agents which may trigger other chronic, demyelinating diseases such as multiple sclerosis. Indeed, circumstantial evidence implicating RNA viruses as possible etiologic agents of multiple sclerosis (6, 66) has stimulated the study of a number of different in vivo and in vitro animal models. Among them are several models involving rodents infected with Theiler's virus (15), Semliki Forest virus (58), measles virus (7), and murine coronavirus (1, 9, 39, 61, 62, 68), each of which displays fundamental differences in its replication strategy, host range, and pathogenesis.

Although a great variety of virus types can produce disease in the CNS, the mechanisms by which latency and persistence are established are only partially understood for a few of the neurotropic DNA and retroviruses belonging to the papovavirus, herpesvirus, and lentivirus groups (for a review, see reference 22). With the above types of agents, formation of DNA copies and provirus intermediates provides the mechanisms for virus perpetuation in an integrated state. However, RNA agents, except for retroviruses, do not replicate via an intermediate DNA phase and, therefore, have no obvious operational mechanism to account for their latency or persistence. Yet, by appropriate manipulation of the systems, such as exposure to neutralizing antibodies, treatment with certain metabolites influencing cell growth and differentiation, and infection with conditional lethal mutants, viruses can be driven into a repressed state (22). For example, metabolites which affect intracellular levels of cyclic AMP (cAMP) have been shown to profoundly affect virus replication in various cell types (4, 37, 48, 75). The effect may be on either the activation of infectious-progeny formation from a virus formerly in a latent state (67) or the suppression of replication (4, 37, 48, 75). With JHM virus

* Corresponding author.

† Present address: Center for Neurobiology and Behaviour, New York, NY 10032.

(JHMV), we have demonstrated that elevation of cAMP concentration, which is coincident with differentiation, irreversibly blocks replication in primary rat and mouse oligodendrocytes (4, 73). This implies that metabolic events leading towards differentiation may be involved in regulating virus expression. In an effort to correlate some aspect of cAMP-induced differentiation with coronavirus suppression in primary rat oligodendrocytes, we directed our attention towards the activation of the cAMP-dependent protein kinase cascades because, according to the current dogma, all effects of cAMP are mediated through these pathways (17, 25).

MATERIALS AND METHODS

Cell culture. L-2 murine fibroblasts (51) and L6 rat myoblasts (74) were routinely propagated as monolayers in M5 nutrient medium (4).

Primary rat neural cell cultures of oligodendrocytes and astrocytes were prepared by a simplification of the method of McCarthy and deVellis (33, 34) described by us previously (4). The cells were grown in 10 ml of Eagle basal medium (BME₁₀) plus 10% fetal bovine serum. To enrich for oligodendrocytes, the cells which had been shaken were allowed to adhere for 24 h and then were released by manual shaking and replated at a final density of 2×10^5 to 3×10^5 cells per cm². Astrocyte cultures were obtained after removal of oligodendrocytes (4).

Virus preparation and methods for inoculating and assaying infectivity. Origin and preparation of mouse hepatitis virus strains MHV₃ and JHMV have been described previously (4, 31).

Infection of neural cells at a multiplicity of infection of 0.5 to 1.0 PFU per cell was carried out in 24-well plates (Nunc 143982). Oligodendrocytes were plated at a density of 4×10^5 to 5×10^5 cells per well, and astrocytes were plated at approximately 1×10^5 to 2×10^5 cells per well. After adsorption for 60 min at 37°C and removal of unattached inoculum, the cultures were incubated with 1 ml BME₁₀.

Assays of coronavirus (in PFU per milliliter) were made on L-2 cell monolayers as described previously (4).

Treatment of cell cultures with inducers of differentiation. Primary neural cells were treated with N⁶,2'-*O*-dibutyryl adenosine-3',5'-cyclic monophosphate (dbcAMP), 8-bromoadenosine-3',5'-cyclic monophosphate (8-Br cAMP), N²,2'-*O*-dibutyrylguanosine-3',5'-cyclic monophosphate (dbcGMP), or papaverine (Sigma Chemical Co., St. Louis, Mo.) or isoproterenol (Isuprel; Winthrop Laboratories, Aurora, Ontario, Canada) or forskolin (Calbiochem-Behring Diagnostics, San Diego, Calif.). The metabolites were diluted in BME₁₀ and applied at various concentrations and for various intervals, depending on the experiment.

Time course studies on the interrelationship between the concentration of the regulatory subunits RI and RII of the cAMP-dependent protein kinases, protein kinase I (PK I) and protein kinase II (PK II), and dbcAMP treatment were carried out as follows. Astrocytes were seeded in BME₁₀ at a density of 5×10^6 cells per plate in 100-mm culture dishes, and oligodendrocytes were seeded at a density of 3×10^6 to 5×10^6 cells per flask in 25-cm² flasks. The medium, plus dbcAMP, was changed daily until the time of harvesting. The duration of exposure to dbcAMP was varied by commencing dbcAMP treatment of duplicate cultures on succeeding days, whereby incubation in the presence of dbcAMP ranged from 0 to 5 days. By staggering the day on which treatment was initiated, it was possible to harvest all the cultures of uniform age simultaneously.

Adsorption and penetration of virus. Oligodendrocyte cultures were seeded onto 35-mm culture dishes for both the adsorption and penetration assays. Cell densities varied as indicated in the results for the adsorption assays. Oligodendrocytes were seeded at a plating density of 1.5×10^6 cells per dish for the penetration assays. Virus was applied at a multiplicity of infection of 10, and all assays were done in triplicate. The procedures used to measure adsorption and uptake have been previously described (16, 67a).

Preparation of cell extracts. To prepare cytosol material for photoaffinity labeling of the protein kinase regulatory subunits and phosphatase assays, 3×10^6 oligodendrocytes or 5×10^6 astrocytes or L6 myoblasts were rinsed twice with ice-cold phosphate-buffered saline, harvested, and centrifuged into pellets at $150 \times g$ at 4°C for 10 min. After suspending in 600 μ l of ice-cold buffer containing 10 mM Tris hydrochloride (pH 7.4), 1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 50 μ g of phenylmethylsulfonyl fluoride per ml, and 2 μ g of leupeptin per ml, the cells were disrupted by applying 40 strokes in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at $100,000 \times g$ for 60 min at 4°C, and the supernatant was designated the cytosol fraction.

The remaining particulate fractions were rinsed gently three to four times with the Tris buffer, then suspended in 500 μ l of the above buffer with 1% Triton X-100 (Tris-Triton), and dispersed by 40 strokes in a Potter-Elvehjem homogenizer to form a homogeneous suspension. The resulting suspensions of cell fragments were stored at -70°C. Protein was determined by the method of Lowry (30).

Measurement of cAMP-dependent protein kinases and [³H] cAMP binding activities. Protein kinase activity and [³H] cAMP binding activity to the regulatory subunits of PK I and PK II were determined by the methods of Roskoski (50) and Gilman (18), respectively, with modifications described elsewhere (49, 57).

Photoaffinity labeling of RI and RII. Covalent linking of the photosensitive cAMP analog, 8-azido-[³²P]cAMP (45 Ci/mM, ICN Pharmaceuticals Inc., Irvine, Calif.), to RI and RII was carried out as described previously (70). After initiating the reaction in a mixture of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.2, 10 mM MgCl₂, 1 mM EGTA, 2 μ g of leupeptin per ml, 50 μ g of phenylmethylsulfonyl fluoride, 25 nM 8-azido-[³²P]cAMP, and 5 μ g of cytosol protein in 100 μ l at room temperature for 60 min in the dark, the samples were irradiated on ice for 10 min by means of an UVS-11 hand lamp, at a distance of 10 cm. Laemmli sodium dodecyl sulfate (SDS)-sample buffer (27) was then added to each reaction mixture in preparation for electrophoresis. The specificity of the coupling reaction was tested by competition in the presence of a 50:1 mixture of cold cAMP and 8-azido-[³²P]cAMP.

Samples were analyzed on 10% polyacrylamide gels with 0.1% SDS by the method of Laemmli (27). Quantitative estimates were made from the autoradiograms by means of a LKB Ultrascan XL laser densitometric scanner.

Assays for phosphatase activity towards JHMV nucleocapsid protein. Phosphoserine-specific phosphatase activity against JHMV nucleocapsid protein N was assayed in a 50- μ l reaction volume containing 25 mM Tris, 25 mM MES (pH 5.5), 5 μ l of ³⁵S- or ³²P-labeled JHMV-infected L-cell extract, 1 mM divalent cations, and 50 μ g of protein from the oligodendrocyte particulate fraction. After incubation for 90 min at 30°C, the reaction was terminated by mixing this 1:1 with sample buffer containing 8 M urea, 20% glycerol, 20% 2-mercaptoethanol, 20 μ l of acetic acid and 10 ml of sample

TABLE 1. Relationship between differentiation inducers and coronavirus replication in primary rat oligodendrocytes^a

| Treatment | Titer (10 ² PFU/ml) on following day after infection: | | |
|------------------|--|------|----------------|
| | 1 | 3 | 10 |
| None (control) | 1.0 | 1.0 | 100 |
| dbcAMP (1 mM) | 0 | 0 | 0 |
| 8-Br cAMP (1 mM) | 0 | 0 | 0 ^b |
| Isoproterenol | | | |
| 20 μM | 1.75 | 0 | 500 |
| 50 μM | 0.15 | 0 | 0 |
| Forskolin | | | |
| 20 μM | 0.3 | 0.05 | 70 |
| 50 μM | 0.25 | 0 | 0 |
| 100 μM | 0.8 | 0 | 0 |

^a Cells were plated at a density of 2×10^5 to 3×10^5 cells per well.

^b Sampled 9 days after infection.

buffer, and 1 μl of carbol fuchsin. After centrifugation for 5 min in an Eppendorf centrifuge, the samples were loaded onto cylindrical gels (36) and subjected to polyacrylamide gel electrophoresis.

Labeling of JHMV proteins. L-2 cell monolayers were infected with JHMV at a multiplicity of infection of 1 PFU per cell until 60% of the cells had formed syncytia and then were incubated in methionine- or PO₄-free medium, containing 2% dialyzed fetal bovine serum, until the infection had progressed to 100% syncytium formation. Labeling was carried out for 30 min with [³⁵S]methionine (800 Ci/mM; New England Nuclear Corp., Boston, Mass.) or ³²P_i (carrier free, 10 mCi/ml; New England Nuclear Corp.) added at a final concentration of 100 μCi/ml. After being labeled, the cultures were rinsed several times with ice-cold phosphate-buffered saline, taken up to 200 μl of Tris-Triton, and disrupted by repeatedly forcing the cells through a 30-gauge needle. Particulate matter was sedimented at $13,000 \times g$ in an Eppendorf centrifuge at 4°C for 5 min. The supernatant was stored at -70°C.

Two-dimensional gel electrophoresis of nucleocapsid protein. N protein and related products generated by the phosphoprotein phosphatase reactions were analyzed by isoelectrofocusing as described previously (41). Basic proteins were resolved by two-dimensional electrophoresis (36).

Two-dimensional tryptic peptide mapping. ³²P-labeled N material was prepared for two-dimensional tryptic peptide mapping as described previously (76). Spots related to N and presumed N products were excised from dried polyacrylamide gels subjected to two-dimensional electrophoresis, transferred to siliconized tubes containing 1 ml of 0.05 M NH₄HCO₃ (pH 8.0) and 50 μg of trypsin pretreated with tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK) (Sigma). After incubation for 24 h at 37°C, the hydrolysates were lyophilized and suspended two to three times in 1 ml of H₂O. The final lyophilysate was suspended to 10 μl in a buffer (pH 1.9) containing acetic acid-formic acid-water (15:5:80). The phosphopeptides were separated on cellulose plates by electrophoresis at 1,000 V for 20 min, followed by ascending chromatography for 1.5 h in solvent systems consisting of either 1-butanol-pyridine-acetic acid-water, (32:5:25:5:20) for 3 h or isobutyric acid-pyridine-acetic acid-butanol-water (65:5:3:2:29) (53).

RESULTS

Relationship between inducers of differentiation and virus replication. In the present study, we examined the effects of treatment with two analogs of 3',5'-cAMP, known to enhance differentiation in primary and continuous cell lines, on the formation of infectious coronavirus progeny. Pretreatment with 1 mM dbcAMP completely inhibited the replication of JHMV in rat oligodendrocytes (Table 1), confirming our previous findings (4). The analog 8-Br cAMP, another known inducer of differentiation in oligodendrocytes (23, 45), also precluded virus production.

We also tested the effects of isoproterenol and forskolin, which increase the intracellular cAMP concentration through their indirect action on the adenylate cyclase system. These compounds were capable of repressing JHMV replication in primary oligodendrocytes (Table 1). Treatment at several concentrations showed that the effect was complete at 50 μM. It is presumed that there is a relationship in oligodendrocyte cultures between the concentration of metabolite added and the rate of adenylate cyclase induction and hence, intracellular accumulation of cAMP. The validity of this assumption has been demonstrated with forskolin in rat cerebral cortical membranes (56), cultures of S₄₉ mouse lymphoma cells (3), and primary rat Schwann cells (60).

To eliminate the possibility that the elevation of cyclic nucleotide concentration, in general, may suppress coronavirus replication, we treated oligodendrocytes with dbcGMP before infection with JHMV and observed that dbcGMP was not inhibitory for JHMV (data not shown), indicating that coronavirus suppression in oligodendrocytes is specifically related to metabolic events affecting cAMP levels.

Previously we demonstrated that 48 h of pretreating oligodendrocytes with dbcAMP completely abolishes JHMV replication, whereas it only marginally affects the replication of MHV₃ in astrocytes (4). To determine whether a prolonged exposure of astrocytes would be more effective, we pretreated primary astrocyte cultures with 1 mM dbcAMP and determined the titers of MHV₃ produced. Pretreatment of astrocytes for as long as 5 days did not diminish the ability of these cells to produce virus (Table 2). This observation is consistent with the view that elevation of cAMP differentially affects coronavirus replication in astrocytes and oligodendrocytes.

Responses of cAMP-dependent protein kinases to modulation of cAMP concentration. To examine the effects of modulating cAMP levels, we first assessed the relative activities of PK I and PK II in untreated neural cell material. For this purpose, cell extracts of astrocytes and oligodendrocytes were prepared, then separated into fractions by DEAE-cellulose column chromatography, and assayed for

TABLE 2. Effect of prolonged exposure to dbcAMP on the replication of MHV₃ in primary rat astrocytes

| Treatment | Titer (10 ² PFU/ml) on following day after infection: | |
|----------------|--|-----|
| | 3 | 5 |
| None (control) | 3.5 | 1.1 |
| dbcAMP (1 mM) | | |
| 1 day | 1.6 | 1.7 |
| 3 days | 1.6 | 2.0 |
| 5 days | 1.6 | 3.2 |

[³H]cAMP binding and kinase activity. Neural cells were conspicuously devoid of PK I (Fig. 1), a finding consistent with the reported absence of the enzyme from freshly isolated neural tissue (71). Here we report for the first time that the predominant isozyme in primary cultures of rat astrocytes and oligodendrocytes was PK II (Fig. 1), which is the enzyme species most abundant in mammalian CNS tissue (40). For comparison with neural cells, protein kinase activity was assayed in L6 myoblasts, a cell line known to restrict coronavirus replication coincident with differentiation (unpublished observations). In confirmation of previous data (49), the L6 line was found to contain considerable amounts of PK I and PK II (data not shown). After treatment with dbcAMP, the activity of PK I in L6 myoblasts was diminished or eliminated completely but that of PK II was not affected. Similarly, there was no effect on PK II levels after 48 h of treatment of neural cell cultures with dbcAMP (unpublished data). It is, therefore, unlikely that virus

expression is contingent upon the presence of PK I per se, because MHV₃ and JHMV were produced in both the L6 cells with PK I and the neural cells without PK I.

Effects of cAMP concentration on the regulatory subunits of PK I and PK II. Changes in RI and RII of astrocytes and oligodendrocytes were monitored after treatment with inducers of differentiation. For this purpose, the binding of RI and RII to either [³H]cAMP or the covalent binding to the photoaffinity analog 8-azido-[³²P]cAMP was determined. For L6 myoblasts, used for comparison, treatment for 40 h with 0.7 mM dbcAMP produced a dissociation of RI from the PK I holoenzyme, thereby generating more of the free form of RI, without alteration in the net amount of RI (data not shown). We also monitored the levels of the regulatory subunit in cytosol extracts of astrocytes and oligodendrocytes by photoaffinity labeling with 8-azido-[³²P]cAMP, which binds specifically to 47,000- and 52,000-molecular-weight polypeptides (47K and 52K polypeptides), shown in previous work to be RI and RII, respectively (48, 69). The binding analysis revealed that treatment of oligodendrocytes with 1 mM dbcAMP for 48 h caused a marked increase in the amount of RI despite the absence of PK I in these cells. By contrast, the quantity of RII appeared to change very little.

The relationship between duration of treatment with 1 mM dbcAMP and the levels of RI and RII in astrocytes and oligodendrocytes was also assessed. The data (Fig. 2; Table 3) show the relative amounts of RI in the densitometric tracing made from the original autoradiogram. In astrocytes treated for 3 days, there was a 2.43-fold increase in RI, but upon extension of the treatment to 5 days, the amount of RI declined to the same level as that in untreated controls. With RII, dbcAMP caused a maximal increase after 3 days to a level only 1.2-fold greater than that present in the untreated controls, declining to 0.61 that of the controls after 5 days of treatment.

For oligodendrocytes, dbcAMP caused a rapid increase in free RI, so that within 24 h this component was about 3.5 times more abundant (Fig. 2; Table 3), and by day 5, RI increased almost 10-fold over the amount present in control cells. By comparison, the level of RII was increased very slowly to 1.48 greater than that in the controls 3 days after treatment and remained unchanged thereafter.

As a test of the specificity of dbcAMP, treatment with 1 mM dbcGMP for 48 h did not cause either an increase or decrease in RI of oligodendrocytes (Fig. 3).

Exposure of oligodendrocytes to 8-Br cAMP suppressed JHMV replication in oligodendrocytes (Table 1). A similar effect was observed with L6 myoblasts (data not shown). This result prompted us to examine the effect of 8-Br cAMP on modulation of the RI subunit. Unexpectedly, treatment of oligodendrocytes with 1 mM 8-Br cAMP caused a decrease of RI compared with the controls (Fig. 3). This finding was also evident in 8-Br cAMP-treated L6 myoblasts (data not shown). As suggested for other systems (64), the reduction in amount of RI in oligodendrocytes may be due to a conformational change produced by the preferential binding of the bromine analog to site 1 of the RI molecule with the resulting destabilization and increased turnover of RI. A similar explanation may be invoked for treatment with forskolin.

The present observations, when taken together, support the idea that inhibition of coronavirus replication in oligodendrocytes is affected through the activation of the adenylate cyclase system manifested in the up or down modulation of free RI.

Early events in host-virus interactions after treatment with

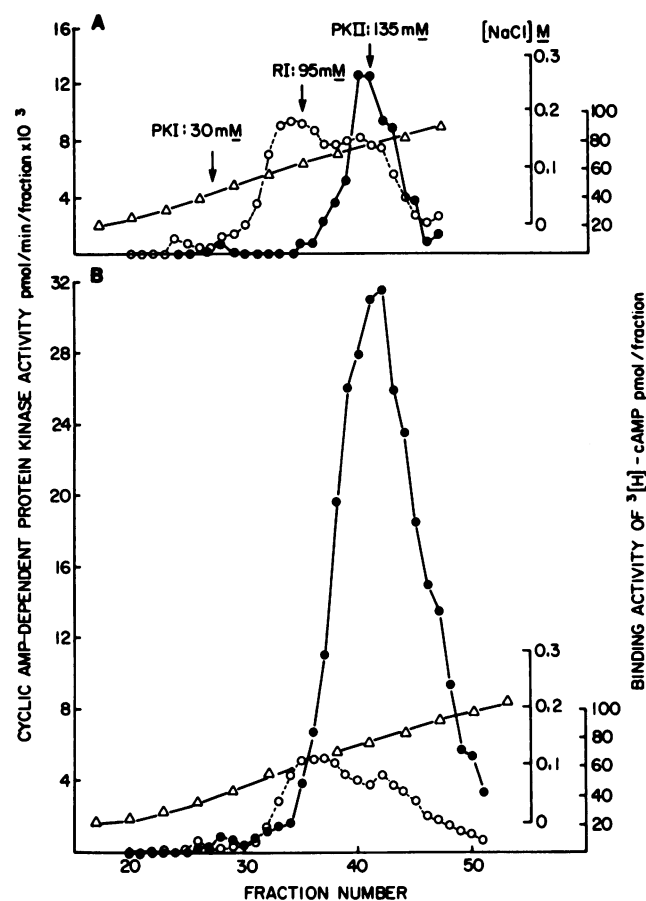


FIG. 1. Chromatography of cAMP-dependent protein kinases and their regulatory subunits in primary neural cells. The cytosol (40,000 × g) component of astrocytes (A) and oligodendrocytes (B) was prepared and separated into 1-ml fractions on DEAE-cellulose, as described in Materials and Methods, and then assayed for cAMP-dependent protein kinases PK I and PK II (●). The data were calculated as the differences between the values obtained with and without 1.5 μM cAMP. The regulatory subunits, RI and RII (○), were detected by binding to [³H]cAMP as described in Materials and Methods. The products were eluted by means of a gradient (Δ) ranging from 0 to 0.4 M NaCl in buffer A. Arrows indicate peak fractions of PK I, RI, and PK II. All activities were normalized for 100 mg of protein loaded.

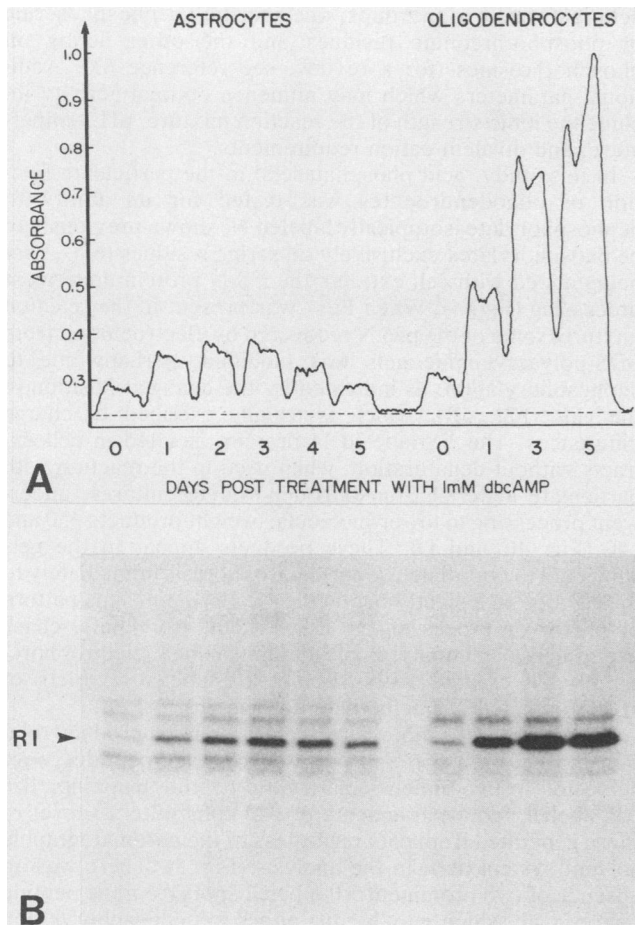


FIG. 2. Modulation of RI and RII in primary rat astrocytes and oligodendrocytes during treatment with 1 mM dbcAMP. The concentrations of the regulatory subunits in cytosol (100,000 × g) fractions from astrocytes and oligodendrocytes were determined by binding of 8-azido-[³²P]cAMP, as described in Materials and Methods. A densitometer tracing (A) made from an autoradiogram (B), obtained after 10% SDS-polyacrylamide gel electrophoresis, enabled a comparison of the time-related changes in the RI regulatory subunit. Absorbance units have been normalized to the band of greatest density (oligodendrocytes, 5 days posttreatment).

inducers of differentiation. To ascertain whether differentiation affects any of the steps in early virus-cell interactions, we examined adsorption, penetration, primary translation, and transcription. Adsorption of JHMV to either astrocytes or oligodendrocytes was unaffected by prior exposure to 1 mM dbcAMP (Table 4). It should be noted from the present

TABLE 3. Modulation of regulatory subunits upon prolonged exposure to dbcAMP in primary rat neural cells

| Cell type | Subunit | Subunit level ^a on following day posttreatment: | | | | | |
|-----------------|---------|--|------|-----------------|------|------|------|
| | | 0 | 1 | 2 | 3 | 4 | 5 |
| Astrocyte | RI | 1.00 | 2.01 | 2.10 | 2.43 | 1.52 | 1.00 |
| | RII | 1.00 | 1.00 | 1.02 | 1.21 | 0.82 | 0.61 |
| Oligodendrocyte | RI | 1.00 | 3.52 | ND ^b | 7.34 | ND | 9.97 |
| | RII | 1.00 | 1.28 | ND | 1.48 | ND | 1.48 |

^a All values, normalized to that on day 0 of each row, are based on densitometer tracings illustrated in Fig. 2.
^b ND, Not determined.

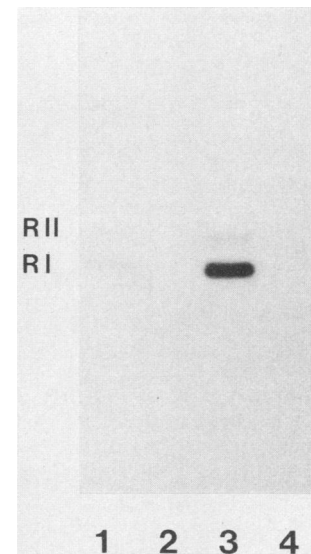


FIG. 3. Modulation of regulatory subunits in primary rat oligodendrocytes after 48 h of exposure to BME₁₀ (lane 1), 1 mM 8-Br cAMP (lane 2), 1 mM dbcAMP (lane 3), and 1 mM dbcGMP (lane 4). Cytosol (100,000 × g) extracts containing 5 μg of protein were reacted with 8-azido-[³²P]cAMP and analyzed on 7.5 to 15% gradient polyacrylamide gel, as described in Materials and Methods.

data that the ability of JHMV to become adsorbed onto astrocytes revealed that specificities in tropism, whereby JHMV fails to replicate in rat astrocytes (4), are not related to the absence of the relevant receptors. Although adsorption frequencies between the cell types varied such that approximately 30% less JHMV became adsorbed to astrocytes, there was no significant difference in the proportion of cells to which virus became complexed.

Similarly, our unpublished data on the rates of JHMV internalization (as defined in Materials and Methods) demonstrated that the capacity by oligodendrocytes to take up the virus was unimpaired, regardless of prior treatment with 1 mM dbcAMP. Virus penetration reached a maximum of 10³ PFU per culture at approximately 15 min after initiation of uptake by warming to 37°C, then gradually fell during the subsequent 2 h, indicating eclipse of the inoculum.

To ascertain whether dbcAMP affected viral transcription and translation in oligodendrocytes, infected cultures were analyzed for JHMV RNA synthesis by dot blots (11, 13, 14) and were analyzed for antigen expression by using antibodies in conjunction with the indirect immunofluorescence technique (4). Although transcription and translation were manifested in untreated oligodendrocytes, after treatment we were unable to detect viral transcripts or primary viral

TABLE 4. Adsorption of JHMV to various cell types of rat origin

| Cell type | PFU | Cell density | Frequency (plaques/cell) |
|------------------|------------------------|---------------------|--------------------------|
| Oligodendrocytes | | | |
| Control | 2.64 × 10 ⁴ | 2 × 10 ⁶ | 1.32 × 10 ⁻² |
| With 1 mM dbcAMP | 2.00 × 10 ⁴ | 2 × 10 ⁶ | 1.0 × 10 ⁻² |
| Astrocytes | | | |
| Control | 2.80 × 10 ³ | 8 × 10 ⁵ | 3.6 × 10 ⁻³ |
| With 1 mM dbcAMP | 2.80 × 10 ³ | 8 × 10 ⁵ | 3.5 × 10 ⁻³ |

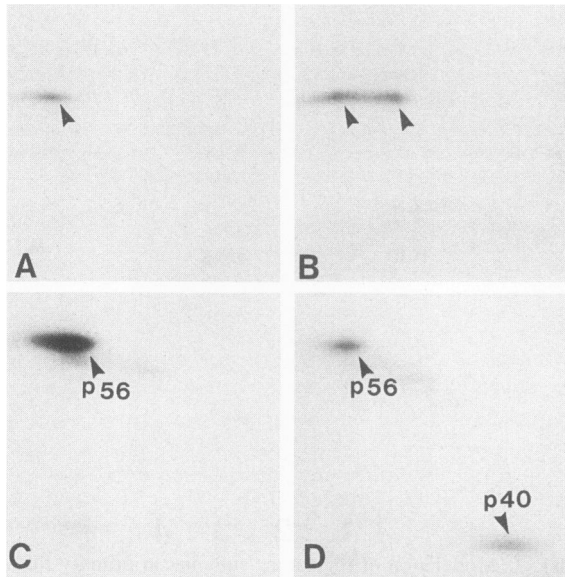


FIG. 4. Processing of JHMV N protein under conditions favorable for Fe^{2+} -dependent acid phosphatase activity. (A and B) Protein (100 μg) from oligodendrocyte particulate material (100,000 $\times g$) was incubated for 90 min at 30°C (pH 5.5) with (A) or without (B) 1 mM Fe^{2+} . Arrowheads indicate the positions of N. In panel B, note the additional spot, presumably arising from a change in charge. To emphasize the spots, the autoradiogram was exposed for 20 (B) or 5 days (A). (C and D) The material used in the reaction, carried out as described above, was [^{35}S]-labeled JHMV N recovered from infected L-2 cells. The reaction was carried out without Fe^{2+} (C); processing to p40 was evident when Fe^{2+} was added (D).

antigens at the level of sensitivity afforded by either technique (data not shown).

Taken together, these results indicate that expression of JHMV within differentiated oligodendrocytes was interrupted at some stage after virion internalization but before initiation of translation or transcription.

Effect of differentiation and acid phosphatase activities in oligodendrocytes. Although the mechanism of coronavirus uncoating has not been elucidated, the fact that in this group the plus-stranded RNA genome is, by itself, infectious (70) implies that viral functions may commence after the genome has been divested of the nucleocapsid protein coat, in which it is wrapped when inside the virus envelope. The presence of phosphorylated residues in N suggested the possibility that uncoating of the RNA, by removal of N from the genome, is facilitated by one or more among the repertoire of host enzymes related to the cAMP-dependent protein kinase cascades. Therefore, repression of these enzymes in differentiated oligodendrocytes might be the underlying cause of arrested JHMV expression. This notion is consistent with a relationship between the induction of cAMP-dependent protein kinase cascades, manifested by modulation of the levels of RI and the inhibition of JHMV expression. It also agrees with the view of Ingebritsen and Cohen (20) that protein phosphatases and kinases are important targets for cellular regulation. On the basis of this background information and evidence for coronavirus penetration via endosomes (26, 38), organelles which possess an acidic environment due to very high activities of a proton ATPase (32), we examined the possible relationship between differentiation and acidic protein phosphatase(s) of oligodendrocytes.

It should be recalled that eucaryotic protein phosphatases

belong to two major groups, one specific for phosphoserine or phosphothreonine residues and the other acting on phosphotyrosines (for a review, see reference 63). Additional parameters which may influence optimal activity include the ionic strength of the reaction mixture, pH, temperature, and divalent cation requirements.

In this study, acid phosphatase(s) in the particulate fraction of oligodendrocytes was tested for its ability to dephosphorylate isotopically labeled N, shown previously to be phosphorylated exclusively on serine residues (65). After being mixed with cell extracts the ^{35}S -N protein underwent processing (Fig. 4). When Fe^{2+} was present in the reaction mixture, some of the p56 N recovered by electroelution from SDS-polyacrylamide gels was modified, perhaps due to dephosphorylation, as indicated by the changed position in the gels (Fig. 4B), which is usually ascribed to charge differences. The ^{35}S -labeled N protein, isolated in cell extracts without denaturation, when used in the reaction with particulate material from oligodendrocyte cultures, underwent processing to lower-molecular-weight products p50 and p40 (Fig. 4C and D). These products appear in the gels subjected to two-dimensional electrophoresis immediately to the right of and slightly below the viral protein. This pattern of precursor processing is also evident from pulse-chase experiments and analyses of one-dimensional electrophoresis of gels treated with SDS of ^{35}S -labeled extracts of JHMV-infected L-2 cells (data not shown).

To demonstrate more directly a precursor-product relationship between p56 and p40, the two polypeptides were subjected to two-dimensional tryptic peptide mapping. The ^{32}P -labeled peptide fragments of p40 constitute a subset of those generated from p56, regardless of the chromatographic solvent system used in the analysis (Fig. 5). There was an absence of two prominent ^{32}P -labeled spots from the peptide map of p40, which may be due either to dephosphorylation

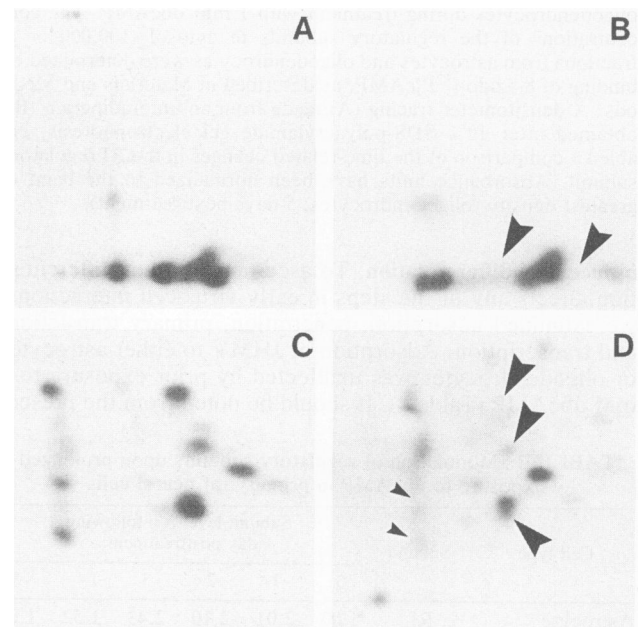


FIG. 5. Two-dimensional phosphotryptic peptide analysis of JHMV N protein and p56 (A and C) and the p40 cleavage product (B and D). Chromatography in two separate solvent systems (A and B versus C and D) demonstrated that p40 is related to p56. Large and small arrowheads indicate the absence of major and minor phosphopeptides, respectively.

or loss of the relevant region as a result of proteolytic cleavage, or as the consequence of both events.

The relationship between treatment of oligodendrocytes with differentiation inducers and activity of N-related phosphatase(s) in the particulate fraction was also examined. It appears that extracts derived from untreated control cultures produced more efficient processing of the ^{32}P -N protein than did comparable extracts from cultures treated with either dbcAMP or 8-Br cAMP, as judged by the quantity of p40 appearing (Fig. 6). These observations support the idea that acid phosphatase(s) affect N processing during normal coronavirus penetration and uncoating, and that this is repressed in differentiated oligodendrocytes. In support of the hypothesis linking processing of N with uncoating, we examined the association of nucleic acids with N by a nucleic acid overlay protein blotting assay as adapted to the coronaviruses (47).

We observed that p56 was the prominent JHMV protein binding nucleic acid, confirming the findings of Robbins et al. (47). It may be highly significant that neither JHMV RNA or cDNA probe was adsorbed to the polypeptide band at the position of p40, indicating that this component lacked the relevant domain for binding nucleic acid (data not shown).

DISCUSSION

The data from this study are consistent with the hypothesis that in primary rat oligodendrocytes, coronavirus replication is suppressed as a consequence of treatment with metabolites that stimulate or mimic increased intracellular cAMP levels. Moreover, suppression of coronaviruses is conditional upon enhancement of an aspect of the adenylate cyclase system which is related to or causes modulation in the amounts of RI. This idea is reinforced by the finding that in primary rat astrocytes, which are relatively uninducible

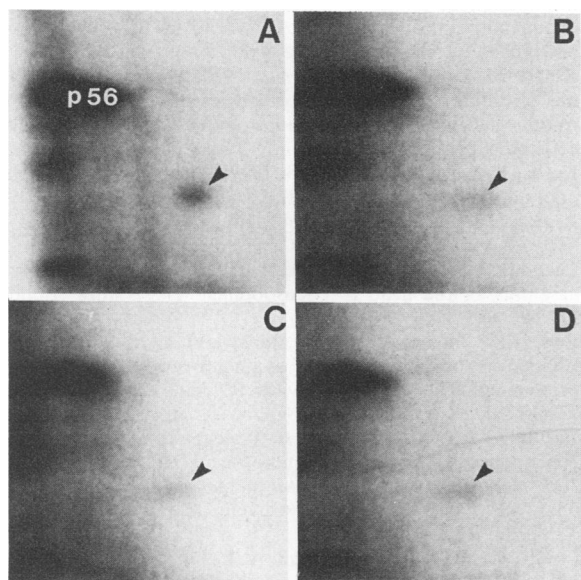


FIG. 6. Two-dimensional gel analysis of processing of ^{32}P -labeled JHMV N under conditions favoring Fe^{2+} -dependent acid phosphatase activity. N material was incubated for 60 min at 30°C with $50\ \mu\text{g}$ of protein from oligodendrocyte particulate fractions ($100,000 \times g$). (A and C) Particulate material from untreated oligodendrocytes incubated with (A) and without (C) $1\ \text{mM}\ \text{Fe}^{2+}$; (B and D) particulate material from oligodendrocytes pretreated with $1\ \text{mM}\ \text{dbcAMP}$ (B) or $1\ \text{mM}\ 8\text{-Br}\ \text{cAMP}$ (D), incubated in the presence of $1\ \text{mM}\ \text{Fe}^{2+}$. Arrowheads indicate the position of p40.

for RI, pretreatment to elevate cAMP does not affect replication of MHV₃. These observations may have a direct bearing on the age-related resistance to JHMV, which develops in preweanling rats (61) and oligodendrocyte cultures in vitro (4), at the time that CNS myelination and oligodendrocyte differentiation are being completed.

In other cell-virus systems, the same differentiation inducers as those used here can either activate or suppress the infection, depending on the particular system being tested (37, 48, 73), emphasizing an apparent linkage between increased intracellular cAMP levels and differentiation and suppression of coronavirus replication. However, in primary rat oligodendrocytes pretreated with dbcAMP, vesicular stomatitis virus and measles virus were reported by us to replicate normally (4), demonstrating that this type of control over virus expression is somewhat specific for each virus group and the particular host cell involved.

Recently, correlations have been made between the induction of differentiation and increased amounts of RI or RII, depending on the cell type under investigation (29, 46, 49, 52, 55, 72). Compounds such as dbcAMP and 8-Br cAMP, when applied before but not after inoculation, produce inhibition of coronavirus transcription and translation without affecting either attachment or sequestration of the inoculum, indicating that a block occurs at some stage after uptake but before expression of genomic functions, drawing attention to the possibility that uncoating is the critical event.

The presumed function of cAMP as a second messenger suggests a connection between regulation of viral functions and differentiation mediated through protein kinase and phosphatase activities. Phosphorylation of nucleocapsid proteins of various RNA agents has been connected with different aspects of virus expression, including the stimulation of influenza virus transcription (21) and the regulation of transcription in vesicular stomatitis virus (19, 24). The possibility that dephosphorylation of N might be required for uncoating, in line with evidence of Leis et al. (28) that dephosphorylation of an avian retrovirus N protein, pp12, reduces the binding affinity between this component and the RNA genome approximately 100-fold prompted us to examine the effects of oligodendrocyte differentiation on cellular phosphatases. This idea is supported by Western blot (immunoblot) evidence which reveals that during penetration, the JHMV N protein of inoculum virions is rapidly converted from a 56K into a 50K component (13). Since the 56K and 50K polypeptides appear to be identical by two-dimensional tryptic peptide mapping (5, 10), reduction in the apparent molecular weight could be due to an alteration in charge, perhaps due to removal of phosphorus. It remains to be resolved whether processing into p50 is initiated by dephosphorylation or proteolytic cleavage, as previously suggested (5, 10), or a combination of the two events. Whether the 50K polypeptide is formed by dephosphorylation or proteolysis may be difficult to prove with ^{35}S methionine-labeled N material because this protein contains 86 amino acids and 13 potential trypsin cleavage sites on the carboxy side of the last methionine residue (59), whereby unlabeled tryptic fragments could be formed. Failure of p40 to bind nucleic acids in the nucleic acid overlay protein blotting assay indicates that the domain for RNA binding has been lost during processing. Inability to detect p40 in extracts of JHMV-infected cells by either Western blot analysis with mouse monoclonal anti-N antibodies (13) suggests that the reactive epitope is missing from p40. Inhibition of N processing by addition of extracts of oligodendrocytes pretreated with $1\ \text{mM}\ \text{dbcAMP}$ or $8\text{-Br}\ \text{cAMP}$ suggests that the

requisite factors are suppressed upon differentiation, coincident with both a reduction in phosphoserine-specific Fe^{2+} -dependent acid phosphatase activity, similar in type to that shown to exist in murine and porcine tissues (42, 43, 54) and a block at an early stage of JHMV expression. Taken together this evidence is in line with our hypothesis that coronavirus uncoating, which is normally facilitated by phosphatase-catalyzed nucleocapsid dephosphorylation, may be the step in processing affected by differentiation.

The importance of protein phosphatases as mediators of cellular regulation has recently received much attention (12, 20, 63). Since virus replication depends on an appropriation of the metabolic machinery of the host, it is not surprising that certain viral functions may be altered or abrogated when oligodendrocytes undergo numerous metabolic changes during differentiation (2, 8, 35, 44, 72). Perhaps among these changes is the modulation of phosphatases which brings about a repression of coronavirus replication by impeding the uncoating process.

Although inhibition of coronavirus replication associated with differentiation does not entirely account for the progress of the in vivo disease (62), it does present an attractive hypothesis for exploring the mechanisms controlling persistent and latent virus infections in the CNS.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council of Canada and the Multiple Sclerosis Society of Canada.

LITERATURE CITED

- Bailey, O. T., A. M. Pappenheimer, F. S. Cheever, and J. B. Daniels. 1949. A murine virus (JHM) causing disseminated encephalomyelitis with extensive destruction of myelin. II. Pathology. *J. Exp. Med.* **90**:195-231.
- Barbarese, E., and S. E. Pfeiffer. 1981. Developmental regulation of myelin basic protein in dispersed cultures. *Proc. Natl. Acad. Sci. USA* **78**:1953-1957.
- Barber, R., and T. J. Goka. 1985. Adenylate cyclase activity as a function of forskolin concentration. *J. Cyclic Nucleotide Res.* **10**:23-29.
- Beushausen, S., and S. Dales. 1985. In vivo and in vitro models of demyelinating disease. XI. Tropism and differentiation regulate the infectious process of coronavirus in primary explants of the rat CNS. *Virology* **141**:89-101.
- Bond, C. W., K. Anderson, and J. L. Leibowitz. 1984. Protein synthesis in cells infected by murine hepatitis virus JHM and A59: tryptic peptide analysis. *Arch. Virol.* **80**:333-347.
- Brody, J. A., J. L. Sever, A. Edgar, and J. McNew. 1972. Measles antibody titres of multiple sclerosis patients and their siblings. *Neurology* **22**:492-499.
- Burnstein, T., J. H. Jensen, and B. H. Waksman. 1964. The development of a neurotropic strain of measles virus in hamsters and mice. *J. Infect. Dis.* **14**:265-272.
- Cammer, W., D. S. Snyder, T. R. Zimmerman, Jr., M. Farooq, and W. T. Norton. 1982. Glycerol phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, and lactate dehydrogenase: activities in oligodendrocytes, neurons, astrocytes, and myelin isolated from developing rat brains. *J. Neurochem.* **38**:360-367.
- Cheever, F. S., J. B. Daniels, A. M. Pappenheimer, and O. T. Bailey. 1949. A murine virus (JHM) causing disseminated encephalomyelitis with extensive destruction of myelin. I. Isolation and biological properties of the virus. *J. Exp. Med.* **90**:181-194.
- Cheley, S., and R. Anderson. 1981. Cellular synthesis and modification of murine hepatitis virus polypeptides. *J. Gen. Virol.* **54**:301-311.
- Cheley, S., and R. Anderson. 1984. A reproducible micro-analytical method for the detection of specific RNA sequences by dot-blot hybridization. *Anal. Biochem.* **137**:15-19.
- Cohen, P. 1982. The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature (London)* **296**:613-620.
- Coulter-Mackie, M., R. Adler, G. Wilson, and S. Dales. 1985. In vivo and in vitro models of demyelinating diseases. XII. Persistence and expression of corona JHM virus functions in RN2-2 schwannoma cells during latency. *Virus Res.* **3**:245-261.
- Coulter-Mackie, M. B., W. C. Bradbury, S. Dales, W. F. Flintoff, and V. L. Morris. 1980. In vivo and in vitro models of demyelinating disease. IV. Isolation of Halle measles virus-specific RNA from BGMK cells and preparation of complementary DNA. *Virology* **102**:327-338.
- Dal Canto, M. C., and H. L. Lipton. 1979. Recurrent demyelination in chronic central nervous system infection produced by Theiler's murine encephalomyelitis virus. *J. Neurol. Sci.* **42**:391-405.
- Dales, S., and H. Hanafusa. 1972. Penetration and intracellular release of the genomes of avian RNA tumor viruses. *Virology* **50**:440-458.
- Flockhart, D. A., and J. D. Corbin. 1982. Regulatory mechanisms in the control of protein kinases. *Crit. Rev. Biochem.* **13**:133-186.
- Gilman, A. G. 1970. A protein binding assay for adenosine 3',5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. USA* **61**:477-483.
- Hsu, C.-H., E. M. Morgan, and D. W. Kingsbury. 1982. Site-specific phosphorylation regulates the transcriptive activity of vesicular stomatitis virus NS protein. *J. Virol.* **43**:104-112.
- Ingebritsen, T. S., and P. Cohen. 1983. Protein phosphatases: properties and role in cellular regulation. *Science* **221**:331-338.
- Kamata, T., and Y. Watanabe. 1977. Role of nucleocapsid protein phosphorylation in the transcription of influenza virus genome. *Nature (London)* **267**:460-462.
- Kauffmann, R. S., and B. N. Fields. 1985. Pathogenesis of viral infections, p. 153-167. In B. N. Fields, D. M. Knipe, R. M. Chanock, J. L. Melnick, B. Roizman, and R. E. Shope (ed.), *Virology*. Raven Press, New York.
- Kim, S. U., G. Moretto, D. H. Shin, and V. M. Lee. 1985. Modulation of antigenic expression in cultured adult human oligodendrocytes by derivatives of adenosine 3',5'-cyclic monophosphate. *J. Neurol. Sci.* **69**:81-91.
- Kingsford, L., and S. U. Emerson. 1980. Transcriptional activities of different phosphorylated species of NS protein purified from vesicular stomatitis virions and cytoplasm of infected cells. *J. Virol.* **33**:1097-1105.
- Krebs, E. G., and J. A. Beavo. 1979. Phosphorylation-dephosphorylation of enzymes. *Annu. Rev. Biochem.* **48**:923-959.
- Krzystyniak, K., and J. M. Dupuy. 1984. Entry of mouse hepatitis virus 3 into cells. *J. Gen. Virol.* **65**:227-231.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Leis, J., S. Johnson, L. S. Collins, and J. A. Traugh. 1984. Effects of phosphorylation of avian retroviruses nucleocapsid protein pp12 on binding of viral RNA. *J. Biol. Chem.* **259**:7726-7732.
- Loeffler, F., S. M. Lohmann, B. Walckhoff, U. Walter, and B. Hamprecht. 1985. Selective increase of RI subunit of cyclic AMP-dependent protein kinase in glial-rich primary cultures upon treatment with dibutyryl cyclic AMP. *Brain Res.* **344**:322-328.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
- Lucas, A., W. Flintoff, R. Anderson, D. Percy, M. Coulter, and S. Dales. 1977. In vivo and in vitro models of demyelinating diseases: tropism of the JHM strain of murine hepatitis virus for cells of glial origin. *Cell* **12**:553-560.
- Marsh, M. 1984. The entry of enveloped viruses into cells by endocytosis. *Biochem. J.* **218**:1-10.
- McCarthy, K. D., and J. deVellis. 1978. Alpha adrenergic receptor modulation of beta-adrenergic, adenosine and pro-

- taglandin E₁-increased adenosine 3':5' cyclic monophosphate levels in primary cultures of glia. *J. Cyclic Nucleotide Res.* **4**:15-26.
34. McCarthy, K. D., and J. deVellis. 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell. Biol.* **85**:890-902.
 35. McMorris, F. A. 1983. Cyclic AMP induction of the myelin enzyme 2',3'-cyclic nucleotide 3'-phosphohydrolase in rat oligodendrocytes. *J. Neurochem.* **41**:506-515.
 36. Mets, L. J., and L. Bogorad. 1974. Two-dimensional polyacrylamide gel electrophoresis: an improved method for ribosomal proteins. *Anal. Biochem.* **57**:200-210.
 37. Miller, C. A., and D. R. Carrigan. 1982. Reversible repression and activation of measles virus infection of neural cells. *Proc. Natl. Acad. Sci. USA* **79**:1629-1633.
 38. Mizzen, L., A. Hilton, S. Cheley, and R. Anderson. 1985. Attenuation of murine coronavirus infection by ammonium chloride. *Virology* **142**:378-388.
 39. Nagashima, K., H. Wege, and V. Ter Meulen. 1978. Early and late CNS effects of coronavirus infection in rats. *Adv. Exp. Med. Biol.* **100**:395-409.
 40. Nairn, A. C., H. C. Hemmings, Jr., and P. Greengard. 1985. Protein kinases in the brain. *Annu. Rev. Biochem.* **54**:931-976.
 41. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007-4021.
 42. Paigen, K. 1958. The properties of particulate phosphoprotein phosphatase. *J. Biol. Chem.* **233**:388-394.
 43. Paigen, K., and S. Griffiths. 1958. The intracellular location of phosphoprotein phosphatase activity. *J. Biol. Chem.* **234**:299-303.
 44. Pfeiffer, S. E., E. Barbarese, and S. Bhat. 1981. Non-coordinate regulation of myelinogenic parameters in primary cultures of dissociated fetal rat brain. *J. Neurosci. Res.* **6**:369-380.
 45. Pleasure, D., J. Parris, J. Stern, J. Grinspan, and S. U. Kim. 1986. Incorporation of tritiated galactose into galactocerebroside by cultured rat oligodendrocytes: effects of cyclic adenosine 3',5'-monophosphate analogues. *J. Neurochem.* **46**:300-302.
 46. Prashad, N., and R. N. Rosenberg. 1978. Induction of cAMP-binding proteins by dibutyryl cyclic AMP in mouse neuroblastoma cells. *Biochem. Biophys. Acta* **539**:459-469.
 47. Robbins, S. G., M. F. Frana, J. J. McGowan, J. F. Boyle, and K. V. Holmes. 1986. RNA-binding proteins of coronavirus MHV: detection of monomeric and multimeric N protein with an RNA overlay-protein blot assay. *Virology* **150**:402-410.
 48. Robbins, S. J., and F. Rapp. 1980. Inhibition of measles virus replication by cyclic AMP. *Virology* **106**:317-326.
 49. Rogers, J. E., S. Narindrasorasak, G. A. Cates, and B. D. Sanwal. 1985. Regulation of protein kinase and its regulatory subunits during skeletal myogenesis. *J. Biol. Chem.* **260**:8002-8007.
 50. Roskoski, R. J. 1983. Assays of protein kinase. *Methods Enzymol.* **99**:3-6.
 51. Rothfels, K. H., A. A. Axelrad, L. Siminovitch, E. A. McCulloch, and R. C. Parker. 1959. The origin of altered cell lines from mouse, monkey and man as indicated by chromosome and transplantation studies. *Proc. Can. Cancer Conf.* **3**:189-214.
 52. Sato, M., A. Hiragun, and H. Mitsui. 1985. Differentiation-associated increases of cAMP-dependent type II protein kinase in a murine preadipose cell line (ST 13). *Biochim. Biophys. Acta* **844**:296-305.
 53. Scheidtmann, K. H. 1986. Phosphorylation of simian virus 40 large T antigen: cytoplasmic and nuclear phosphorylation sites differ in their metabolic stability. *Virology* **150**:85-95.
 54. Schlosnagle, D. C., E. G. Sander, F. W. Bazer, and R. M. Roberts. 1976. Requirement of an essential thiol group and ferric ion for the activity of the progesterone-induced porcine uterine purple phosphatase. *J. Biol. Chem.* **251**:4680-4685.
 55. Schwartz, D. A., and C. S. Rubin. 1983. Regulation of cAMP-dependent protein kinase subunit levels in Friend erythro-leukemic cells. *J. Biol. Chem.* **258**:777-784.
 56. Seamon, K. B., and J. W. Daly. 1981. Forskolin: a unique diterpene activator of cAMP generating systems. *J. Cyclic Nucleotide Res.* **7**:201-224.
 57. Seth, P. K., J. Rogers, S. Narindrasorasak, and B. D. Sanwal. 1983. Regulation of adenosine 3':5' monophosphate phosphodiesterases: altered pattern in transformed myoblasts. *J. Cell Physiol.* **116**:336-344.
 58. Sheahan, B. J., P. N. Barret, and G. J. Atkins. 1981. Demyelination in mice resulting from infection with a mutant of Semliki forest virus. *Acta Neuropathol.* **53**:129-136.
 59. Skinner, M. A., and S. G. Siddell. 1983. Coronavirus JHM: nucleotide sequence of the mRNA that encodes nucleocapsid protein. *Nucleic. Acid. Res.* **11**:5045-5054.
 60. Sobue, G., S. Shumann, and D. Pleasure. 1986. Schwann cell responses to cyclic AMP: proliferation, change in shape, and appearance of surface galactocerebroside. *Brain Res.* **362**:23-32.
 61. Sorensen, O., D. Percy, and S. Dales. 1980. In vivo and in vitro models of demyelinating diseases. III. JHM virus infection of rats. *Arch. Neurol.* **37**:478-484.
 62. Sorensen, O., A. Saravani, and S. Dales. 1987. In vivo and in vitro models of demyelinating disease. XVII. The infectious process in immunosuppressed or athymic rats inoculated with JHM virus. *Microbial Pathogenesis* **2**:79-90.
 63. Sparks, J. W., and D. L. Brautigan. 1986. Molecular basis for substrate specificity of protein kinases and phosphatases. *Int. J. Biochem.* **18**:497-504.
 64. Steinberg, R. A., and D. A. Agard. 1981. Turnover of regulatory subunit of cAMP-dependent protein kinase in S₄₉ mouse lymphoma cells. *J. Biol. Chem.* **256**:10731-10734.
 65. Stohlman, S. A., and M. M. C. Lai. 1979. Phosphoproteins of murine hepatitis viruses. *J. Virol.* **32**:672-675.
 66. Tanaka, R., V. Iwasaki, and H. Koprowski. 1976. Intracisternal virus-like particles in brain of a multiple sclerosis patient. *J. Neurol. Sci.* **28**:121-126.
 67. Van Alstyne, D., and D. W. Paty. 1983. The effect of dibutyryl cyclic AMP on restricted replication of rubella virus in rat glial cells in culture. *Virology* **124**:173-180.
 - 67a. Van Dinter, S., and W. F. Flintoff. 1987. Rat glial C6 cells are defective in murine coronavirus internalization. *J. Gen. Virol.* **68**:1677-1685.
 68. Virelizier, J. L., A. D. Dayan, and A. C. Allison. 1975. Neuro-pathological effects of persistent infection of mice by mouse hepatitis virus. *Infect. Immun.* **12**:1127-1140.
 69. Walter, U., M. R. Costa, X. O. Breakfield, and P. Greengard. 1979. Presence of free cyclic AMP receptor protein and regulation of its level by cAMP in neuroblastoma-glioma hybrid cells. *Proc. Natl. Acad. Sci. USA* **76**:3251-3255.
 70. Wege, H., A. Muller, and V. ter Meulen. 1978. Genomic RNA of the murine coronavirus JHM. *J. Gen. Virol.* **41**:217-227.
 71. Weldon, S. L., M. C. Mumby, and S. C. Taylor. 1985. The regulatory subunit of neural cAMP-dependent protein kinase II represents a unique gene product. *J. Biol. Chem.* **260**:6440-6448.
 72. Wernicke, J. F., and J. J. Volpe. 1986. Glial differentiation in dissociated cell cultures of neonatal rat brain: non-coordinate and density-dependent regulation of oligodendroglial enzymes. *J. Neurosci. Res.* **15**:39-47.
 73. Wilson, G. A. R., S. Beushausen, and S. Dales. 1986. In vivo and in vitro models of demyelinating diseases. XV. Differentiation influences the regulation of coronavirus infection in primary explants of mouse CNS. *Virology* **151**:253-264.
 74. Yaffe, D. 1968. Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. USA* **61**:477-483.
 75. Yoshikawa, Y., and K. Yamanouchi. 1984. Effect of papaverine treatment on replication of measles virus in human neural and nonneural cells. *J. Virol.* **50**:489-496.
 76. Zweig, S. E., and S. J. Singer. 1979. The two components of spectrin, filamin, and the heavy chain of smooth muscle myosin show no detectable homologies to one another by two-dimensional mapping of iodinated tryptic peptides. *Biochem. Biophys. Res. Commun.* **88**:1147-1152.