Novel 2',5'-Oligoadenylates Synthesized in Interferon-Treated, Vaccinia Virus-Infected Cells

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2-5A[ppp(A2'p)$_n$A] and related materials accumulate to greater than micromolar concentrations in vaccinia virus-infected HeLa cells and in interferon-treated, vaccinia-infected HeLa, CV1, and L929 cells even when virus replication is not inhibited. A variably complex mixture of authentic 2-5A (n = 2 to 4), nonphosphorylated cores [(A2'p)$_n$A; n = 2 to 5], and additional compounds of unknown structure was observed.

Modulation of the 2-5A system is one of the many effects of interferon (IFN) treatment of animal cells (reviewed in reference 13). The 2-5A synthetase, when activated by double-stranded RNA, polymerizes ATP into a series of 2',5'-linked oligomers [ppp(A2'p)$_n$A, $n \geq 2$]. These oligomers, collectively called 2-5A, bind and activate a latent cellular endoribonuclease (the 2-5A-dependent RNase, RNase L or F) capable of cleaving mRNA and rRNA. As well as synthesizing 2-5A, the synthetase can add AMP in 2'-5' linkage to such metabolites as NAD$^+$, ADP-ribose, and A5'p$_4$5'A (1, 5, 7, 14). Although these products have not been found in intact cells (5), other unusual 2',5' oligoadenylates have been detected, the structure and function of which have not yet been determined (4, 10).

Vaccinia virus replication is extremely sensitive to IFN in some strains of L cells grown in suspension in which protein synthesis, both cellular and viral, is rapidly inhibited. Activation of the 2-5A-dependent RNase with cleavage of rRNA and viral RNA has been reported to correlate with this general inhibition of protein synthesis (2, 6, 8). In contrast, in other cell lines, vaccinia replication is relatively resistant to IFN and there is no correlation between 2-5A levels and inhibition of virus growth (15, 17). In L929, HeLa, and CV1 cells we observed extraordinarily high (greater than micromolar) levels of 2-5A after vaccinia infection even when the cells were treated with too little IFN to inhibit virus growth. Similar high levels of 2-5A accumulated in untreated HeLa cells (which have high constitutive levels of 2-5A synthetase) in which vaccinia replicates well (17). A partial explanation for this apparent anomaly was provided by the observation that in this system activation of the 2-5A-dependent RNase in the intact cell lags considerably behind the accumulation of 2-5A. The enzyme itself, however, remained active in cell extracts prepared throughout infection, suggesting the presence of an inhibitor(s) in the intact cell (17). Initial results in our laboratory indicated that, in addition to authentic 2-5A, a variety of related compounds were present in substantial amounts. It seemed possible that a further analysis of this 2-5A-related material might reveal the presence of compounds capable of inhibiting

activation of the RNase (i.e., analog inhibitors) or, alternatively, reveal products indicative of an alternative function(s) for the 2-5A system. The results of such an analysis are presented here.

HeLa cells (S3), L929 cells, and CV1 cells were grown in monolayer culture with Dulbecco modified Eagle medium supplemented with 10% heat-inactivated newborn calf serum. IFN treatment for 20 h before infection was done with 400 or 800 reference units of human (Namalva) cell IFN (Wellcome Research Laboratories; >10^8 reference units per mg of protein) for the HeLa and CV1 cells, respectively, and with mouse L-cell IFN (>5 × 10^7 reference units per mg of protein) for the L929 cells. Infection with crude vaccinia virus (strain WR) was at 10 PFU per cell (17). Trichloroacetic acid (TCA)-soluble extracts were prepared from culture dishes of cells at the indicated time points. In some cases the extracts were desalted and concentrated on a C18 Sep-Pak column (Waters Associates) (4). Bacterial alkaline phosphatase (BAP; Sigma type III-R) digestions to remove terminal phosphate groups were performed at 37°C overnight at 0.25 mg/ml (final enzyme concentration) and were monitored by the addition of tracer amounts of ppp(A2'p)$_3$A[^{32}P]pCp (1 × 10^3 to 6 × 10^3 Ci/mmol; Amersham Corp.). High-pressure liquid chromatography (HPLC) was done on µBondapak C18 columns (Waters Associates) using a gradient of methanol (0 to 12.5% from 0 to 25 min, 12.5 to 25% from 25 to 35 min, and 25% thereafter) in 80 mM ammonium phosphate buffer, pH 7.0. In this HPLC system (3) the 5' diphosphates [pp(A2'p)$_n$A] elute slightly more slowly and are not completely resolved from their respective 5' triphosphates [ppp(A2'p)$_n$A]. The 5' monophosphates [p(A2'p)$_n$A] are well resolved. "Cores" [(A2'p)$_n$A] elute late and are poorly resolved (e.g., see Fig. 3A, peak J). For optimum resolution of cores a potassium phosphate-methanol gradient was used (3). The radiobinding (RB), radioimmune (RI), and rRNA cleavage assays were as described previously (11, 12, 17). Of the assays used the rRNA cleavage and RB assays are the more specific for authentic 2-5A. Each requires the minimum structure pp(A2'p)$_2$A for optimum sensitivity (<1 nM). For the RI assay, which uses a polyclonal antiserum, the corresponding minimum structure is p(A2'p)$_2$A (11). All of the assays, however, detect additional 2',5' oligoadenylates with reduced sensitivity, e.g., the RI assay detects structures containing pA2'pA at 20 to 60 nM (11), and the rRNA cleavage assay detects the tetramer 5'-monophosphate p(A2'p)$_3$A at 100 nM (9). For simplicity, only the

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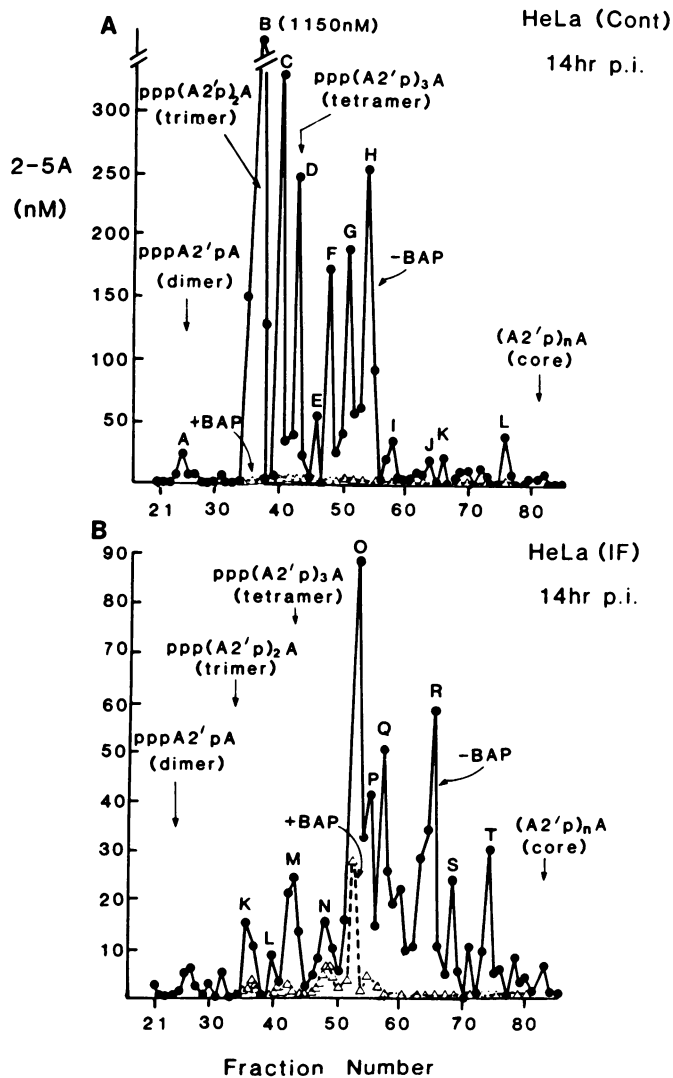
FIG. 1. HPLC analysis of 2′,5′ oligoadenylates extracted from control and IFN-treated HeLa cells at 14 h p.i. with vaccinia virus. TCA-soluble extracts from control (A) and IFN-treated (B) vaccinia virus-infected HeLa cells were analyzed by HPLC with (△) or without (●) prior BAP digestion. The HPLC fractions were diluted and assayed by the RB assay. The amounts loaded (measured by the RB assay) were 500 (A) and 280 (B) pmoles undigested and 220 (A) and 250 (B) pmoles with prior BAP digestion (but measured before digestion). Concentrations are given in terms of that concentration of 2-5A (expressed in AMP equivalents) which would have given the same displacement as the unknown in the RB assay. The positions to which dimer, trimer, tetramer, and core standard 2-5A migrated in parallel analyses are indicated.

results with the RB and rRNA cleavage assays are presented here.

TCA-soluble extracts from HeLa cells obtained 14 h postinfection (p.i.) were fractionated by HPLC with and without prior BAP digestion and assayed by the RB assay (Fig. 1A). Typical results for an rRNA cleavage assay on the non-BAP-digested material are presented in Fig. 2. Twelve peaks of RB-positive material were detected (Fig. 1A). Of these, B and D are presumably trimer and tetramer 2-5A [(p)pp(A2′p)$_n$A, n = 2 and 3]. They eluted identically to the corresponding standards and activated the 2-5A-dependent

RNase with activities consistent with the results of the RB assays. Peaks A, C, and E to L (Fig. 1A) cannot be ascribed unambiguous structures; only C, E, F, G, and H activated the RNase (Fig. 2). E or F could be pentamer [(p)pp (A2′p)$_4$A]. No RB-positive material was obtained after BAP digestion (Fig. 1A). Thus all 12 peaks in the undigested material (Fig. 1A) probably contained 5′ phosphates. Similar results with this material were obtained with the RI assay (data not shown). It is noteworthy (in contrast to the results with CV1 cells, see below) that negligible (<10 nM) nonphosphorylated core material was detected in the RI assay in the undigested material (Fig. 1A).

Very different results were obtained upon infection of the same cells after IFN treatment (Fig. 1B). Once again peaks (K and M, Fig. 1B) presumably corresponding to trimer and tetramer 2-5A were identified as described above. The remaining peaks (L and N to T) cannot be ascribed structures; of these, only O was positive in the rRNA cleavage assay (controls in which 2-5A was added to the assays failed to detect inhibitory material). The significance of the residual RB activity in the BAP digest, despite complete digestion of the internal standard ppp(A2′p)$_3$A[^{32}P]pCp to (A2′p)$_3$-A[^{32}P]pC, is unclear.

Complex patterns of 2′,5′ oligoadenylates were also obtained from IFN-treated, vaccinia virus-infected CV1 cells. A particularly complex example is shown in Fig. 3 without or with prior BAP digestion (Fig. 3A and B, respectively). In addition to peaks of material positive in RB and rRNA cleavage assays which eluted at positions corresponding to trimer, tetramer, and pentamer 2-5A (Fig. 3A, peaks A, B, and C), a variety of additional peaks was detected. These appeared to differ substantially from those present in the corresponding HeLa extract (Fig. 1B). The major differences are that with the CV1 material (peaks D to I) the values measured in the RI assay were high compared to the values measured in the RB assay, and these peaks were active in the rRNA cleavage assays at levels consistent with their RB values (contrast peaks P to T in Fig. 1B). In addition, the CV1 material yielded many RB-positive peaks after BAP digestion (Fig. 3B) and contained a major peak J without BAP digestion (Fig. 3A) at a position corresponding to the core standard. This peak contained enough material (9 μM in the RI assay) to rerun in an alternative HPLC system (see above and reference 3) monitoring A_{260}. This confirmed that the material consisted of nonphosphorylated trimer, tetramer, pentamer, and hexamer core 2-5A [(A2′p)$_n$A, n = 2 to 5, data not shown].

There was, in fact, considerable variation in 2-5A synthetase levels and quantitative and qualitative differences in the complexity of the pattern of 2′,5′ oligoadenylates obtained with CV1 cell lines of different origins. For example, the level of total RB-positive material at 23 h p.i. in IFN-treated cells varied from 60 nM to 30 μM (in 2-5A equivalents). Culture conditions also appeared to greatly affect the results. In general, sparse cultures gave substantially lower levels of 2′,5′ oligoadenylates in cells than did confluent cultures. As might be expected the pattern also varied with time p.i.

Simpler patterns were obtained in a less extensive analysis of 2′,5′ oligoadenylates in IFN-treated L929 cells 12 h p.i. with vaccinia. Four peaks of RB-positive material were obtained, all of which were BAP sensitive. Only two were active in the rRNA cleavage assay, and these eluted in positions consistent with their being trimer and tetramer 2-5A. In general, much less RB-positive material was obtained from these cells.
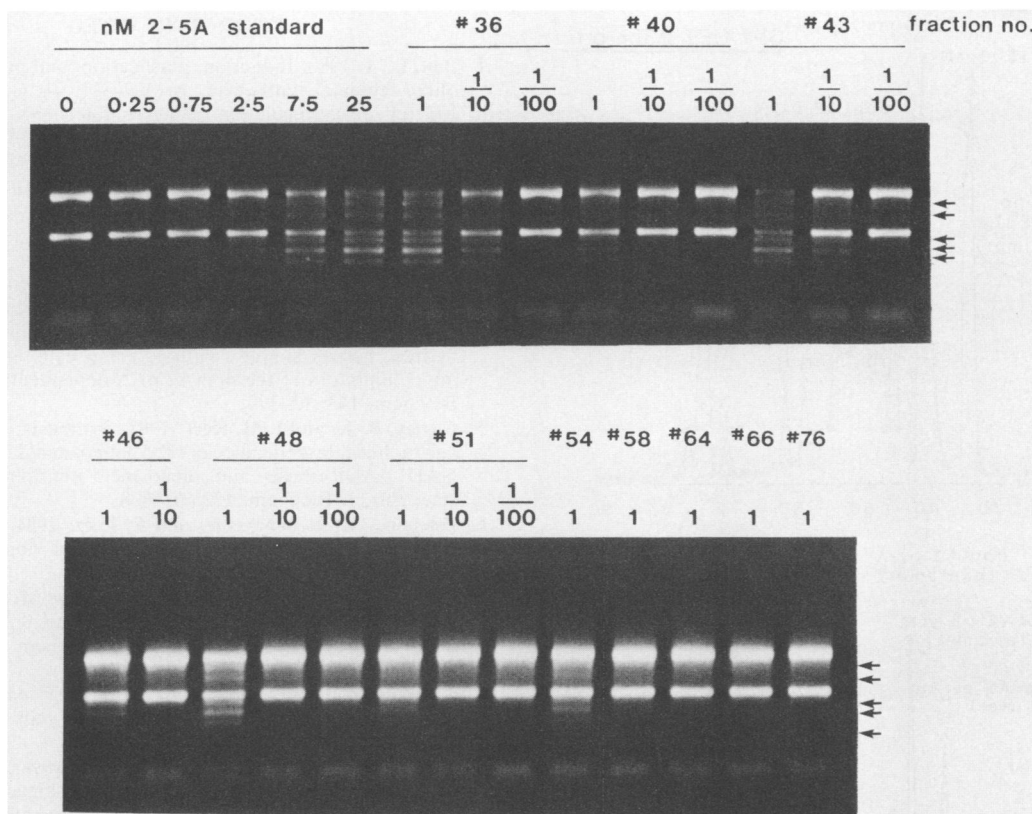
FIG. 2. Activation of 2-5A-dependent RNase by HPLC-fractionated 2′,5′ oligoadenylates extracted from control HeLa cells at 14 h p.i. with vaccinia virus. Peak samples from the undigested material analyzed in Fig. 1A were incubated with an IFN-treated L-cell cytoplasmic extract (S-10). RNA isolated from such incubations was electrophoresed in a 1.6% neutral agarose gel (17); a photograph of the ethidium bromide-stained gel is shown. Specific 2-5A-dependent RNase cleavage products of 28S and 18S rRNAs are marked by arrows to the right of the gel. The top left portion of the gel contained incubations with 0, 0.25, 0.75, 2.5, 7.5, and 25 nM concentrations (final concentration in AMP equivalents) of a ppp(A2′p)₃A standard. The fraction numbers above the gel correspond to those shown in Fig. 1A. Tracks labeled 1/10 or 1/100 indicate that corresponding dilutions of the HPLC fractions were assayed. All incubations (8 μl) included 2 μl of sample and 6 μl of S-10.

This analysis has shown that 2′,5′ oligoadenylates in vaccinia-infected cells can consist of a variably complex mixture of authentic 2-5A, nonphosphorylated core 2-5A, and numerous additional compounds of unknown structure. These findings contrast with those in other IFN-virus systems. In IFN-treated, encephalomyocarditis virus-infected L cells, only authentic 2-5A (trimer and tetramer) and cores were detected (12). Little authentic 2-5A was detected in CV1 cells infected with simian virus 40 and then treated with IFN, but core 2-5A and several oligoadenylates of unknown structure were found (10). In IFN-treated, herpes simplex virus type 1-infected Chang cells, authentic 2-5A (trimer and tetramer) was detected as well as two related compounds which were capable of acting as inhibitors of the 2-5A-dependent RNase in cell extracts (4). These related compounds were present in concentrations too low to permit further characterization (4). It is not clear whether any of the unknowns in the systems described above correspond to any of those detected in the present study.

The reason(s) for the lag in vaccinia-infected cells between accumulation of high levels of 2-5A and activation of the RNase (17) remains unclear. In our study, assay of selected peak fractions (not themselves capable of activating the RNase) failed to detect significant analog inhibitory activity analogous to that found in IFN-treated, herpesvirus-infected cells (4). It remains possible that such inhibitors are produced at an early or crucial time during infection or are localized at high concentrations near the RNase. Alternatively, another type of inhibitor of the RNase that is not related to 2-5A could be present which is lost during the preparation of cell extracts.

Clearly, much of the variability in the amount and nature of the 2′,5′ oligoadenylates in vaccinia-infected cells reflects differences between cell lines. Even within a given cell line, however, the results were so variable as to suggest extreme sensitivity to time and multiplicity of infection, variation in the IFN response and the exact growth state of the cells. With cells yielding sufficient 2′,5′ oligoadenylates for further characterization to appear possible a priori, the quantitative and qualitative variation from experiment to experiment meant that none of the unknowns could be reproducibly obtained in sufficient yield for structural determination. Accordingly, no further analysis was attempted. The nature and role of the additional material, therefore, remains unclear. It cannot be excluded that these compounds are produced by the fortuitous modification of 2-5A by vaccinia-encoded enzymes (e.g., capping enzyme; see reference 16) and are not of widespread biological relevance. On the other hand, authentic 2-5A is produced in relatively large amounts in a variety of IFN-
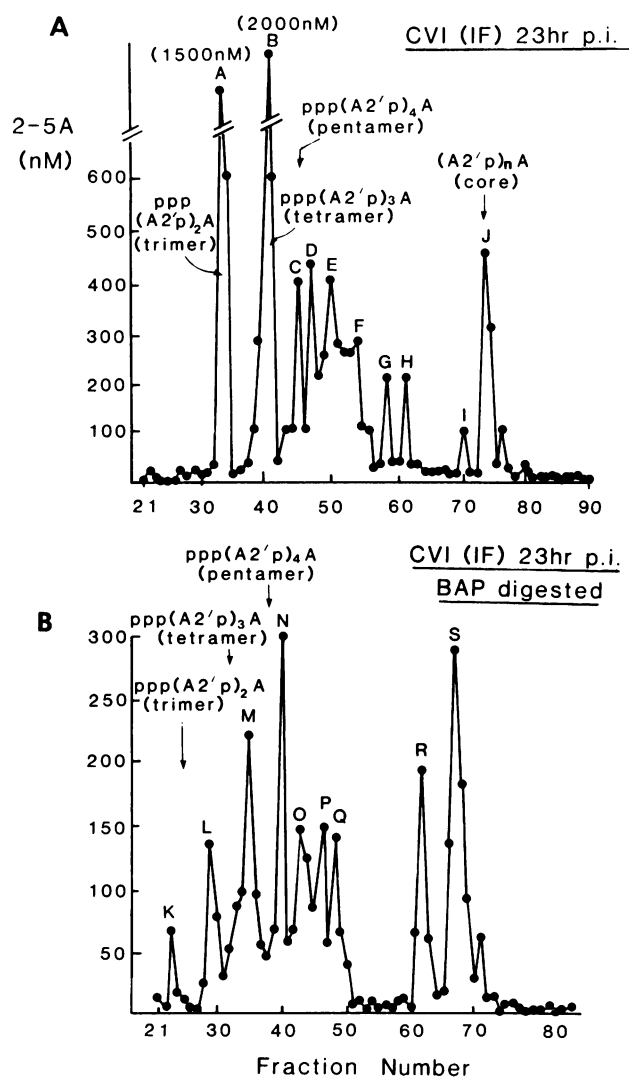
FIG. 3. HPLC analysis of 2',5' oligoadenylates extracted from IFN-treated CV1 cells at 23 h p.i. with vaccinia virus. Samples were incubated without (A) and with (B) BAP before HPLC fractionation. Individual HPLC fractions were assayed by the RB assay. Total amounts of 2',5' oligoadenylates loaded measured by the RB assay were 6,000 pmol (A) and 4,500 pmol (B) (measured before digestion). Peaks are labeled in order of elution. Concentrations and standards are as in Fig. 1.

treated, vaccinia virus-infected cells in which the 2-5A-dependent RNase is not activated until late in infection. In the absence of any detectable inactivation of the RNase per se, the mechanism of inhibition of its activation by 2-5A remains to be established.

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