Involvement of the influenza A virus PB2 protein in the regulation of viral gene expression

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To determine the function(s) of the PB2 protein of influenza A virus, six temperature-sensitive (ts) mutants of A/Udorn/72 (H3N2) virus, each carrying a ts mutation in the PB2 gene, were analysed for virus RNA and protein synthesis. One of the mutants, ICRC27, exhibited unique phenotypes and was characterized in detail. At the non-permissive temperature, 40 °C, the accumulation of mRNA for each genome segment was reduced severely, leading to delayed and reduced synthesis of viral proteins, complementary and viral RNAs (cRNAs and vRNAs). At the permissive temperature, 34 °C, the mutant virus produced several-fold greater concentrations of both mRNAs and cRNAs of PB2, PB1 and PA segments than wild-type virus. The synthesis of the three polymerase proteins and the induction of RNA polymerase activity were also greatly increased. By contrast, the expression of the haemagglutinin (HA) gene was severely suppressed. The over-production of the polymerase mRNAs was not observed during primary transcription, i.e. in the presence of cycloheximide. The ts revertants of ICRC27 did not exhibit the ts defects and also lost most of the non-ts phenotypes at 34 °C. These observations indicate that the PB2 protein participates not only in the synthesis of viral RNAs, but also in the regulation of viral gene expression, i.e. in the down-regulation of the three polymerase genes and the up-regulation of the HA gene during secondary transcription.

Introduction

The genome of influenza A virus comprises eight ssRNA segments of negative polarity (Lamb & Choppin, 1983). In virus-infected cells, the genomic RNAs (vRNAs) are transcribed into two different types of RNA, mRNA and complementary RNA (cRNA; the templates for vRNA synthesis) (Hay et al., 1977a; for a review see McCauley & Mahy, 1983). Viral mRNA synthesis in infected cells is catalysed by a complex of three polymerase proteins, PB1, PB2 and PA, which is carried in the virion, being associated with viral nucleocapsids (Horisberger, 1980; Ulmanen et al., 1981; Kawakami & Ishihama, 1983). The virion polymerase is responsible for primary transcription (Hay et al., 1977b; Barrett et al., 1979). Viral transcription is initiated at the 3' end of a primer, which is a capped oligonucleotide of 10 to 13 bases generated from host cell heterogeneous nuclear RNA by the vRNA polymerase complex (Bouloy et al., 1978; Plotch et al., 1979; Beaton & Krug, 1981; Ulmanen et al., 1983; Kawakami et al., 1983). The PB2 protein has been shown to have a site(s) that recognizes and binds to the type 1 cap structure of the heterogeneous nuclear RNA (Braam et al., 1983). By contrast, less is known about the mechanism of subsequent steps of replication.

Several studies have been carried out to reveal the relationship between the synthesis of viral RNAs and proteins (Lamb & Choppin, 1976; Hay et al., 1977b; Inglis & Mahy, 1979; Barrett et al., 1979; Smith & Hay, 1982; Enami et al., 1985; Shapiro et al., 1987; Hatada et al., 1989). In MDCK cells infected with A/Udorn/72 (H3N2) virus, the synthesis of virus-specific RNAs proceeds as follows (Hatada et al., 1989). During primary transcription at early times of infection, considerable amounts of mRNA are produced from the non-structural protein (NS), nucleoprotein (NP) and the three polymerase genes (early proteins), but very low amounts from the haemagglutinin (HA), neuraminidase (NA) and matrix protein (M) genes (late proteins). At 2 to 3 h post-infection (p.i.), synthesis of vRNA as well as cRNA from all segments begins simultaneously, the cRNA levels for all segments immediately reaching a low plateau, whereas the vRNA levels steadily increase. The syn-
thesis of mRNAs encoding the late proteins starts with the increase in cRNAs and vRNAs. Amounts of all mRNAs, except those from the three polymerase genes, reach a plateau 3 to 4 h p.i., depending on the gene. By contrast, the accumulation of mRNAs encoding the three polymerases ceases 2 h p.i. and is kept at a low level thereafter; their secondary transcription is suppressed to a low level.

To study further the mechanism of viral gene expression, we have analysed temperature-sensitive (ts) mutants of influenza A/Udorn/72 (H3N2) virus (Shimizu et al., 1982a,b; Hatada et al., 1990). Using six ts mutants of the PB2 gene, we studied the synthesis of viral RNAs and proteins in infected MDCK cells to identify the functional roles of the PB2 protein in viral RNA synthesis and its regulation.

Methods

Viruses and cell cultures. Strains of influenza A virus used in this study were derivatives of A/Udorn/72 (H3N2) virus, the wild-type (wt) virus, six ts mutants (UV257, ICR1397, ICR27, SPC44, SP571 and ICR348) of complementation group A having a ts lesion in the PB2 gene and three ts+ revertant viruses of ICR27. The isolation and characterization of the ts mutants have been described previously (Shimizu et al., 1982a, b). Table 1 shows the complementation indexes of these viruses, determined by analysis of crosses with prototypes of eight recombination groups

<table>
<thead>
<tr>
<th>Mutant</th>
<th>A (PB2)</th>
<th>B (PB1)</th>
<th>C (PA)</th>
<th>D (HA)</th>
<th>E (NA)</th>
<th>F (NP)</th>
<th>G (M)</th>
<th>H (NS)</th>
</tr>
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<tbody>
<tr>
<td>UV257</td>
<td>&lt;0.01</td>
<td>1.7</td>
<td>3.2</td>
<td>4.8</td>
<td>1.9</td>
<td>4.7</td>
<td>4.1</td>
<td>4.0</td>
</tr>
<tr>
<td>ICR1397</td>
<td>&lt;0.01</td>
<td>5.6</td>
<td>5.5</td>
<td>3.3</td>
<td>7.8</td>
<td>14.0</td>
<td>7.1</td>
<td>12.0</td>
</tr>
<tr>
<td>ICR27</td>
<td>&lt;0.01</td>
<td>5.8</td>
<td>4.9</td>
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<td>2.6</td>
<td>7.3</td>
<td>2.4</td>
<td>8.9</td>
</tr>
<tr>
<td>SPC44</td>
<td>&lt;0.01</td>
<td>0.4</td>
<td>0.5</td>
<td>1.6</td>
<td>1.4</td>
<td>0.2</td>
<td>5.3</td>
<td>4.1</td>
</tr>
<tr>
<td>SP571</td>
<td>&lt;0.01</td>
<td>2.9</td>
<td>0.3</td>
<td>2.1</td>
<td>0.6</td>
<td>3.5</td>
<td>11.0</td>
<td>2.9</td>
</tr>
<tr>
<td>ICR348</td>
<td>&lt;0.01</td>
<td>1.0</td>
<td>7.6</td>
<td>3.3</td>
<td>1.6</td>
<td>2.1</td>
<td>2.6</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* The complementation index was defined as the ratio of the number of plaques observed at 40 °C to the expected number of cells infected with the two viruses, as described previously (Shimizu et al., 1982b).

The reaction mixture contained, in a final volume of 100 µl, 50 mM-Tris-HCl pH 7.8, 150 mM-NaCl, 5 mM-MgCl₂, 2.5 mM-DTT, 0.25 mM-A-pG or 1 µg reovirus mRNA [prepared as described by Bouloy et al. (1979) and kindly provided by Dr K. Mizumoto], 0.5 mM each of ATP, GTP and CTP, 0.05 mM-[3H]UTP (2 µCi/100 µl), 0.05% NP40 and 20 µg of purified virions. After 1 h incubation at 30 °C or 35 °C, the reaction was terminated by adding TCA to 5% and the acid-insoluble radioactivity was counted.

For the in vitro assay of RNA-dependent RNA polymerase activity in permeabilized cells, infected MDCK cells were harvested by treatment with trypsin, washed twice with TGS buffer (25 mM-Tris-HCl pH 7.4, 140 mM-NaCl, 5 mM-KCl, 0.7 mM-Na₂HPO₄, 5.6 mM-glucose), treated with 1 mg/ml of lysolecithin at 0 °C for 10 min and then washed twice with TGS buffer. The transcription reaction with these permeabilized cells was carried out according to the method described by Beaton & Krug (1986) using 0.25 mM-A-pG or 1 µg/100 µl reovirus mRNA as primer. Primer-dependent RNA polymerase activity was calculated as the value obtained after subtraction of the activity without added primer.

Analysis of viral protein synthesis. Confluent monolayers of MDCK cells on six-well Costar plates were inoculated with 100 µl of virus at a multiplicity of 5 p.f.u./cell for 1 h at room temperature, overlaid with 1 ml of prewarmed MEM, and incubated at either 34 °C or 40 °C. At various times p.i. the medium was replaced with 0.5 ml of methionine-free MEM supplemented with [35S]methionine (4 µCi/ml; specific activity, 1.1 Ci/~µmol) and incubation was continued for a further 30 min. The cells were washed three times with 1.5 ml of TGS buffer. To analyse total protein synthesis, the cells were lysed directly in the dishes by adding 100 µl of an electrophoresis sample buffer containing 62.5 mM-Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol and 10% glycerol. To immunoprecipitate viral proteins, the cells were disrupted by adding 100 µl of an electrophoresis sample buffer containing 62.5 mM-Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol and 10% glycerol. To immunoprecipitate viral proteins, the cells were disrupted by adding 100 µl of lysis buffer (50 mM-Tris–HCl pH 7.5, 0.6 M-KCl, 0.5% NP40). Immunoprecipitation was carried out according to the method described by Kida et al. (1982). Total cell proteins or immunoprecipitates were analysed by electrophoresis on 6 cm 18% polyacrylamide gels containing 0.072% bisacrylamide, 0.1% SDS and 3 M-urea, using the discontinuous buffer system described by Laemmli (1970). The acrylamide:bisacrylamide ratio (250:1) was determined by using the formula of Blattler et al. (1972). After electrophoresis for 2.5 h at 100 V, protein gels were stained with Coomassie brilliant blue and dried for autoradiography.
10 mM-Tris–HCl pH 7.4, 0.15 M-NaCl, 0.3% SDS and 0.4 mg/ml protease K, and incubated at 56°C for 15 min. LiCl buffer (0.2 ml, 10 x 1 M-sodium acetate pH 4.9, 14 M-LiCl, 5% SDS) was then added and the mixture was extracted with 2 ml of water-saturated phenol by shaking it for 5 min at 56°C and further with 2 ml of chloroform for 15 min at room temperature. Nucleic acids were precipitated with ethanol and digested with 30 units of DNase I (Worthington; RNase-free DPFF) for 30 min at 30°C. The resulting RNA was extracted again with phenol–chloroform. A quantitative hybridization system was employed to measure the amount of the three types of virus-specific RNA (mRNA, cRNA and vRNA) for each of the eight genome segments as reported elsewhere (Hatada et al., 1989), in which RNA probes intensely labelled with 32p were used in a molar excess sufficient to overpower complementary RNAs present in the viral RNA samples. The probes for mRNA and cRNA were obtained by transcribing SP 6 recombinant DNAs, which gave the intact 5' end sequences of vRNAs.

As mRNAs have 16 nucleotides at the 5' end of the vRNA deleted, the RNA hybrids formed with mRNAs are shorter than the cRNA hybrids. We can thus discriminate mRNAs from cRNAs by PAGE. Usually, 200 μg of RNA was obtained from 1 x 10^7 cells/85 mm dish and 2 μg of RNA (equivalent to 1 x 10^5 cells) was hybridized with 100 fmol probe. After digestion with RNase T1, hybrids were heat-denatured at 95°C in 80% formamide and then subjected to electrophoresis on 6% polyacrylamide gels containing 7 M-urea (0.5 x 450 x 200 mm in size). The quantity of each hybrid was determined by measuring the intensity of the signal on the autoradiograms of the gels with a microdensitometer. The number of individual viral RNA molecules was estimated from the quantity of hybrid by comparison with those of 5 fmol (= 3 x 10^9) molecules of the corresponding complementary probes.

Results

Temperature sensitivity of virion RNA polymerase activity

For this study, we chose six stable PB2 ts mutants (UV257, ICR1397, ICRC27, SPC44, SP571 and ICR348) from the collection of ts mutants derived from influenza A/Udorn/72 (H3N2) virus (Shimizu et al., 1982a, b). As the PB2 protein is a constituent of the virus transcriptase complex, we analysed the effect of reaction temperature on in vitro transcription catalysed by virion-associated RNA-dependent RNA polymerase. The Ap-G- and reovirus mRNA-primed RNA synthesis was performed at various temperatures using purified virions of the six PB2 ts mutants. The optimum temperature for RNA synthesis by wt virus was 30°C and was greatly reduced at higher temperatures. Wt virus exhibited approximately 70% of the maximum activity at 35°C and only 20% at 40°C, which is the non-permissive temperature used in the initial selection of these ts mutants. For this reason, the temperature sensitivity of the virion RNA polymerase reaction was judged from the ratio of the activity at 35°C to that at 30°C. The temperature sensitivity of ICR1397 was severe and that of SPC44 was considerable, independently of either A-pG or reovirus mRNA, whereas the temperature sensitivity of the other four mutants was similar to that of the wt virus (Table 2).

Virion RNA polymerase of influenza A virus becomes thermostable in the presence of NP40 (Kawakami et al., 1981). To determine whether the PB2 protein is involved in this thermostability, virions were preincubated at 37°C for 10 min in the reaction mixture, which contained 0.05% NP40, but not the substrates. RNA synthesis was initiated by adding substrates and the activity remaining was determined (Table 3). The wt virus retained approximately 80% of the initial activity in either the A-pG- or reovirus mRNA-primed reaction. ICR1397, ICRC27 and SPC44 lost approximately 80% of the activity, whereas UV257, SP571 and ICR348 lost 40 to 60%.

Protein synthesis in virus-infected cells

Protein synthesis in virus-infected cells was analysed by pulse-labelling proteins with [35S]methionine at various times after virus infection. Fig. 1 shows proteins synthesized in MDCK cells between 6 and 6.5 h p.i. with wt virus and the six PB2 ts mutants at permissive (34°C) and non-permissive (40°C) temperatures. In ICR1397-infected cells (lane 3), the synthesis of all viral proteins was barely detectable at 40°C. In cells infected with

| Table 2. Temperature sensitivity of RNA polymerase activity of PB2 ts mutants in vitro |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | 30°C (%) | 35°C (%) | 30°C (%) | 35°C (%) |
| Virus           | A-pG primer | Reovirus mRNA primer | A-pG primer | Reovirus mRNA primer |
| Wt              | 89.6     | 64.5     | 20.7     | 17.6     |
| UV257           | 58.5     | 35.1     | 16.2     | 11.6     |
| ICR1397         | 29.9     | 6.6      | 4.8      | 1.4      |
| ICRC27          | 37.7     | 27.5     | 9.3      | 6.5      |
| SPC44           | 38.9     | 16.0     | 9.7      | 5.6      |
| SP571           | 43.3     | 26.0     | 7.9      | 5.9      |
| ICR348          | 26.5     | 17.7     | 7.9      | 5.5      |

| Table 3. Thermostability of RNA polymerase activity of PB2 ts mutants in vitro |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Activity remaining after preincubation at 37°C for 10 min in the presence of 0.05% NP40 (%) |
| Virus           | A-pG primer | Reovirus mRNA primer |
| Wt              | 79         | 78         |
| UV257           | 42         | 37         |
| ICR1397         | 11         | 15         |
| ICRC27          | 16         | 24         |
| SPC44           | 14         | 24         |
| SP571           | 60         | 48         |
| ICR348          | 57         | 42         |
UV257, ICRC27 and SPC44 (lanes 2, 4 and 5, respectively), the synthesis of the late proteins, HA and M, was reduced at 40 °C compared to that at 34 °C. ICRC27 exhibited abnormal protein synthesis even at 34 °C; the synthesis of the PB1, PA and PB2 proteins increased, whereas the synthesis of HA decreased. With the remaining two strains, SP571 and ICR348 (lanes 6 and 7, respectively), viral protein synthesis was not affected appreciably at either temperature. Analysis of viral proteins after immunoprecipitation with anti-A/Udorn/72 virion antiserum confirmed these observations (data not shown).

The rate of synthesis of viral proteins in ICRC27-infected cells was examined in detail (Fig. 2). At 34 °C the synthesis of the PB1, PA and PB2 proteins was much greater than that of wt virus. In addition, maximal synthesis of viral proteins was delayed 2 h and the level of HA decreased severely. The delay in viral protein synthesis was more extensive at 40 °C and maximal synthesis occurred 7 h p.i., more than 4 h later than that of wt virus. Synthesis of HA and M was still low at 7 h p.i. at 40°C. In ICRC27-infected cells, therefore, the regulation of virus protein synthesis was impaired at both 40 °C and 34 °C. These observations indicate that the PB2 protein is involved in temporal and quantitative control of viral genome expression.

Table 4. Primer-dependent RNA polymerase activity of infected cells in vitro

<table>
<thead>
<tr>
<th>Virus</th>
<th>A-pG primer</th>
<th>Reovirus mRNA primer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34 °C</td>
<td>40 °C (%)</td>
</tr>
<tr>
<td>Wt</td>
<td>43.9</td>
<td>18.5 (42)</td>
</tr>
<tr>
<td>UV257</td>
<td>40.1</td>
<td>0.2 (1)</td>
</tr>
<tr>
<td>ICR1397</td>
<td>35.8</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>ICRC27</td>
<td>310.5</td>
<td>8.6 (3)</td>
</tr>
<tr>
<td>SPC44</td>
<td>60.1</td>
<td>1.0 (2)</td>
</tr>
<tr>
<td>SP571</td>
<td>28.9</td>
<td>5.0 (17)</td>
</tr>
<tr>
<td>ICR348</td>
<td>85.8</td>
<td>1.8 (2)</td>
</tr>
</tbody>
</table>

Induction of RNA polymerase activity in virus-infected cells

Influenza virus-infected cells which are permeabilized by treatment with lysolecithin can catalyse the synthesis of both viral mRNAs and cRNAs in vitro (Beaton & Krug, 1986). By using this method, the induction of viral RNA-dependent RNA polymerase activity was measured for MDCK cells which had been infected with the ts mutant viruses for 6 h at 34 °C or 40 °C (Table 4). Less than 3% of the activity detected at 34 °C was detected at 40 °C with all the ts mutants except SP571,
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Fig. 2. Time course of viral protein synthesis. MDCK cells were infected with ICRC27 (a) or wt virus (b). After virus adsorption, cells were incubated at 34 °C or 40 °C. At 0, 1, 3, 5 and 7 h p.i. (lanes 1 to 5) proteins were labelled for 30 min with [35S]methionine. The labelled proteins were electrophoresed on an 18% SDS-polyacrylamide gel.

whereas approximately 40% was detected with wt virus and 10 to 20% with SP571. Since UV257, ICRC27, SPC44 and ICR348 exhibited the same rate of synthesis of the early proteins, including the three polymerase proteins, at 40 °C as at 34 °C (Fig. 1), these mutants seemed to be defective in the formation of the active transcription complex at 40 °C, although the instability shown in Table 3 could presumably account for the reduced activity.

The most remarkable finding was that cells infected with ICRC27 at 34 °C exhibited sevenfold higher activity than wt virus-infected cells with A-pG primer, and threefold higher activity with reovirus mRNA primer (Table 4). No such elevated activity could be detected in permeabilized cells infected with the other PB2 mutants, nor for RNA synthesis in vitro catalysed by ICRC27 virion-associated RNA polymerase (Table 2). This increased activity coincided with the increased synthesis of the PB1, PA and PB2 proteins in ICRC27-infected cells at 34 °C, as described above.

Viral RNA synthesis in ICRC27-infected cells

Virus-specific RNA synthesis in ICRC27-infected cells was analysed using the quantitative hybridization method (Fig. 3 and Table 5). In wt virus-infected cells there was a burst of synthesis of all viral RNAs and proteins between 2 and 3 h p.i.; mRNA synthesis from the PB2, PB1 and PA genes, and cRNA synthesis from all genome segments was suppressed after this burst to a level of 300 to 1000 copies/cell for each RNA, whereas the accumulation of other mRNAs ceased 3 to 4 h p.i., at a level of 3000 to 20000 copies/cell depending on the genome segment. On the other hand, the level of all vRNAs continued to increase until 7 h p.i., yielding more than 25000 copies/cell.

In ICRC27-infected cells at 34 °C, the burst was delayed for 1 h. The accumulation of both ICRC27 mRNAs and cRNAs from the PB2, PB1 and PA genes was between 1500 and 3200 copies/cell, two- to fourfold and five- to eightfold for mRNA and cRNA more than that in wt virus-infected cells (Table 5). The accumulation of the other viral mRNAs and cRNAs, however, did not exceed that from wt virus. In particular, accumulation of HA mRNA was significantly less than that of wt virus. It was noteworthy that the accumulation of mRNA for the other late proteins, NA and M, was not reduced as much. The level of vRNA except for those for the three polymerase genes decreased at 34 °C.

In ICRC27-infected cells at 40 °C, the burst was delayed about 4 h as judged from the time course of protein synthesis (Fig. 2). Accordingly, only small amounts of viral RNAs were detected at 7 h p.i. The mRNAs, cRNAs and vRNAs for the HA and M genes
Table 5. Viral RNA accumulation in virus-infected cells at 34°C over 5 h

<table>
<thead>
<tr>
<th>Segment</th>
<th>Copy number per cell* (×10⁻³)</th>
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<tbody>
<tr>
<td></td>
<td>mRNA</td>
</tr>
<tr>
<td></td>
<td>Wt</td>
</tr>
<tr>
<td>PB2</td>
<td>0.8</td>
</tr>
<tr>
<td>PB1</td>
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<td>PA</td>
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<td>HA</td>
<td>5.5</td>
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<tr>
<td>M</td>
<td>14.1</td>
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<tr>
<td>NS</td>
<td>21.8</td>
</tr>
</tbody>
</table>

* The intensity of the signals on the autoradiograms shown in Fig. 3 was measured using a microdensitometer and the number of viral RNA molecules was estimated as described in Methods.
† The ratio of the copy number for ICRC27 to that for wt virus.

The late virus genes were barely detectable (Fig. 3) and significant accumulation of cRNAs was observed only for the PB2 and NP genes.

These results indicate that the abnormal pattern of viral protein synthesis in ICRC27-infected cells resulted from the abnormal synthesis of the respective viral mRNAs. To determine whether the over-production of the three polymerase mRNAs was attributable to primary transcription, the cells were infected at 34°C in the presence of 100 μg/ml cycloheximide, a potent inhibitor of protein synthesis. In wt virus-infected cells, mRNAs for the early protein genes (PB2, PB1, PA, NP and NS) only were detected in the presence of cycloheximide, as reported elsewhere (Hatada et al., 1989), and the synthesis of both cRNAs and vRNAs was completely undetectable, as described by Mark et al. (1979) (Fig. 4, data shown for mRNAs and cRNAs). In ICRC27-infected cells in the presence of the drug, transcripts of all the early genes were detected, but they accumulated to significantly lower levels than in wt virus-infected cells. The levels of the three polymerase mRNAs were not increased compared to the other early mRNAs and the corresponding wt mRNAs. These observations indicate that the increased synthesis of the three polymerase mRNAs is not attributable to primary transcription, but to secondary transcription of these genes.

Fig. 3. Time course of viral RNA accumulation in virus-infected cells. MDCK cells were infected with wt virus (W) or ICRC27 (T). After virus adsorption, cells were incubated at 34°C (a and c) or 40°C (b and d). At 3, 4, 5 and 7 h p.i. (lanes 1 to 4), total RNA was extracted and hybridized with a minus-strand RNA probe (a and b) or a plus-strand RNA probe (c and d) to detect the viral mRNAs and cRNAs, or the viral vRNAs, respectively. After digestion with RNase T1, hybrids were analysed by electrophoresis on 6% polyacrylamide gels containing 7 M-urea. The parts of the gel autoradiograms corresponding to viral RNAs are shown. The positions of cRNAs (↑), mRNAs (►) and vRNAs (▼) are indicated for each genome segment. The extra bands seen in the HA, NA and NS rows in Fig. 3, 4 and 6 were presumably derived from the respective mRNA (a and b) or vRNA (c and d) hybrids by nibbling or incomplete digestion, and were considered to be a signal for the mRNA or vRNA.
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Fig. 4. Effect of cycloheximide on viral RNA accumulation in virus-infected cells. MDCK cells were infected with wt virus (a) or ICRC27 (b) in the presence (+CM) or absence (-CM) of 100 μg/ml of cycloheximide and incubated at 34 °C for 3, 5, 7 or 9 h (lanes 1 to 4). Viral RNA accumulation was analysed as described in the legend to Fig. 3.

Phenotypes of ts+ revertant viruses isolated from ICRC27 mutant virus

As described above, ICRC27 exhibited three distinct phenotypes: (i) extensively delayed synthesis of all viral proteins and RNAs at 40 °C; (ii) increased expression of the three polymerase genes at 34 °C; (iii) reduced expression of the HA gene at 34 °C. To study the relationship between these mutational phenotypes, three independent spontaneous ts+ revertants were isolated from ICRC27. The frequency of reversion was in the order of 10⁻⁴. Analysis of protein (Fig. 5) and RNA (Fig. 6) synthesis in revertant-infected cells revealed that these revertants did not exhibit the ts phenotype or the non-ts

Fig. 5. Protein synthesis in ts+ revertant-infected MDCK cells. MDCK cells were infected with wt virus (W), ICRC27 (T), three independent ts+ revertants of ICRC27 (1, 2 and 3) or were mock-infected (C) at either 34 °C (a) or 40 °C (b) for 6 h and proteins were pulse-labelled with [³⁵S]methionine for 30 min. The labelled proteins were analysed by 18% SDS-PAGE.
phenotypes, although considerable over-production of
the three polymerase mRNAs was still observed at 34 °C.
This means that the ts mutation in the PB2 gene of
ICRC27 is related to all the mutant phenotypes. At
40 °C, the over-production of the three polymerase
mRNAs was greater with the revertants, but the level of
cRNA became normal (Fig. 6). Synthesis of the HA
mRNA was excessive at 34 °C, but was reduced at 40 °C.
These observations indicate that some abnormality in
the regulation of the three polymerase and the HA genes
still remained, and that the reversions were not likely to
be true back mutations, but phenotypic suppressor
mutations.

Discussion

In this paper we have analysed the defects exhibited by
six ts mutants of A/Udorn/72 (H3N2) virus, all belong-
ing to recombination group A, which has a mutation(s) in
the PB2 gene segment (Shimizu et al., 1982b). It was
shown that the thermostability of the virion RNA
polymerase of all these mutants was decreased (Table 3).
In addition, the virion enzyme of ICR1397 was
temperature-sensitive in a transcription reaction primed
with either A-pG or reovirus mRNA (Table 2). The PB2
protein is an essential constituent of the transcriptase
complex and has been shown to interact with the type 1
cap structure of host cell heterogeneous nuclear RNA,
utilizing it as a primer for mRNA synthesis (Ulmanen et
al., 1981, 1983; Braam et al., 1983). With the PB2
mutants tested, A-pG-primed transcription, in which
cap recognition by RNA polymerase is not required, was
affected to the same degree as reovirus mRNA-primed
transcription, suggesting that the PB2 protein is
also involved in the transcription process at some stage
other than the cap interaction, as reported previously
(Scholtissee & Bowles, 1975; Krug et al., 1975; Sugiura
et al., 1975; Nichol et al., 1981; Ghendon et al., 1982).

One of the PB2 mutants, ICRC27, exhibited multiple
abnormal phenotypes and, therefore, was subjected to
further investigation. The time course of protein syn-
thesis in ICRC27-infected cells was delayed for 2 h at
34 °C and for more than 4 h at 40 °C compared to that in
wt virus-infected cells. This is due to the inefficiency of
primary mRNA synthesis, as shown by the decreased
level of viral mRNA accumulation in the presence of
cycloheximide in ICRC27-infected cells.

Interestingly, maximum synthesis of PB1, PA and PB2
proteins in ICRC27-infected cells at 34 °C was several-
fold greater than that in wt virus-infected cells. A similar
PB2 mutant in which a high level of polymerase proteins
accumulated has been reported previously (Scholtissee &
Bowles, 1975). However, analysis of viral RNA synthesis
was not studied. The remarkably high RNA synthesis
activity observed with ICRC27-infected cells at 34 °C in
vitro is attributable to this over-production of the
polymerase proteins. Analysis of virus-specific RNAs in
ICRC27-infected cells revealed that the over-production
of the polymerase did not cause over-production of the
virus RNAs in vivo, except for the mRNAs and cRNAs
of the three polymerase genes. This indicates that the
rate of viral RNA synthesis in infected cells is not a
linear function of the amount of RNA polymerase in the
cells. The cessation of the synthesis of mRNAs and
cRNAs despite continued synthesis of vRNAs at late
times of wt virus infection (Hatada et al., 1989) also
suggests a control mechanism for viral RNA synthesis
which selectively suppresses the viral mRNA and cRNA
synthesis to a certain level depending on the genome
segment.

In ICRC27-infected cells at 34 °C, the accumulation of
the mRNAs and cRNAs of the polymerase genes was
two- to fourfold and five- to eightfold greater, respect-
ively, than in wt virus-infected cells, suggesting that the
control mechanism was impaired or modified in
ICRC27. The over-production of the polymerase
mRNAs, however, was not observed in the presence of
cycloheximide, which abolishes protein synthesis. Likewise, selective enhancement of transcription of the polymerase genes was not observed in the transcription reaction in vitro using disrupted ICRC27 virions. The over-production of polymerase mRNAs is, therefore, attributable to an enhancement of their synthesis at secondary transcription. It is conceivable that ICRC27 is defective in a regulatory function(s) at 34 °C, which suppresses the secondary transcription of the three polymerase genes, and that the PB2 protein participates in this down-regulation.

Over-production of cRNAs of the three polymerase genes was also observed in ICRC27-infected cells at 34 °C. Therefore we suggest that transcription of mRNAs and cRNAs from vRNAs for the PB2, PB1 and PA gene segments is controlled as a group and that the PB2 protein participates in this regulation. One possible mechanism for such regulation could be that RNA polymerase functions as a repressor molecule operating at the transcriptional level (Fukuda et al., 1978).

In contrast to the over-expression of the polymerase genes, the expression of the HA gene was severely suppressed in ICRC27-infected cells at 34 °C. In wt virus infection, the expression of the HA gene was suppressed during primary transcription at early times of infection, but increased during secondary transcription at late times of infection. ICRC27 seemed to be defective in the relief of suppression at 34 °C, which should take place at the time of transition from the early to late phase of infection, suggesting that the PB2 protein participates in this up-regulation of HA gene expression.

Three independently isolated ts+ revertants of ICRC27 did not exhibit the ts defects in mRNA synthesis, or the non-ts defects in the regulation of the three polymerase genes and HA gene, presumably by an additional suppressor mutation. Therefore, the two functional sites seem to be intimately interrelated.

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three P polypeptides, from RNA produced by primary transcription. 
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