Increased amounts of the influenza virus nucleoprotein do not promote higher levels of viral genome replication

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INTRODUCTION

The influenza A virus genome consists of eight single-stranded, negative-sense viral genomic RNA (vRNA) segments that associate with the viral polymerase complex (PB1, PB2 and PA) and nucleoprotein (NP) to form ribonucleoproteins (RNPs) (reviewed by Portela & Digard, 2002). After entry into the nucleus of an infected cell (Herz et al., 1981), these segments act as templates to direct the synthesis of two types of positive-sense RNA (reviewed by Elton et al., 2002). First, mRNA is synthesized through a primer-dependent process in which 5'-capped RNA fragments of 10–15 nt are cleaved from host-cell pre-mRNAs and used to prime virus transcription (Plotch et al., 1981). These transcripts are polyadenylated at their 3' ends when the viral polymerase stutters over a polyuridine stretch that is found 15–22 nt from the 5' end of the vRNA (Hay et al., 1977a; Robertson et al., 1981; Poon et al., 1998). The resulting transcripts, containing 5' and 3' modifications, are imperfect copies of their vRNA templates. As such, they cannot serve as substrates to replicate further vRNA molecules. Replication of the viral genome is achieved through primer-independent synthesis of a full-length, positive-sense replicative intermediate, cRNA, that is not polyadenylated (Hay et al., 1982). Whilst the molecular details of viral mRNA synthesis have been described relatively well, the mechanisms by which cRNA is synthesized and the balance between viral transcription and genome replication is regulated remain unclear (reviewed by Elton et al., 2002).

In vitro lysis of purified virus releases transcriptionally competent RNPs that, given the appropriate substrates, synthesize capped and polyadenylated mRNAs (Plotch & Krug, 1977; Plotch et al., 1981). However, the same RNPs do not synthesize cRNA except in the context of an infected cell and then only after a round of mRNA transcription and subsequent protein expression (Hay et al., 1977b; Skorko et al., 1991). Whilst it is possible that cellular factors are necessary to render the input RNPs competent for replication and candidate proteins have been identified (Momose et al., 2001, 2002), definitive proof of such a mechanism is lacking. However, it is well-established that the viral NP is essential for genome synthesis. Several NP temperature-sensitive (ts) mutants have been isolated that are defective for replicative transcription at the non-permissive temperature (reviewed by Portela & Digard,
and some deliberate NP mutations affect genome replication and mRNA transcription differentially (Mena et al., 1999). Furthermore, several studies have shown that, in contrast to detergent-disrupted virus, nuclear extracts from infected cells catalyse both viral mRNA synthesis and genome replication (Beaton & Krug, 1984; del Rio et al., 1985; Takeuchi et al., 1987). Depletion of the fraction of NP that is not already bound into RNPs from these extracts abolishes both c- and vRNA synthesis (Beaton & Krug, 1986; Shapiro & Krug, 1988). Nevertheless, the reasons that NP is required for genome replication remain uncertain. Both v- and cRNA are encapsidated to form functional RNPs (Pons, 1971; Hay et al., 1977b), so this provides part of the explanation (Shapiro & Krug, 1988). Functional analysis of two ts NP mutants that are specifically defective for genome replication supports this hypothesis, because both mutants lose the ability to bind RNA at the non-permissive temperature (Medcalf et al., 1999). However, NP may also play a direct regulatory role in controlling genome replication. Such a role would be particularly likely for positive-strand RNA synthesis, where two types of RNA, differing in their mode of transcription initiation and termination, are made from the same template. One possibility is that newly synthesized NP alters the structure of the RNA promoter sequences found at the termini of each genome segment, thereby biasing the polymerase towards replicative transcription (Hsu et al., 1987; Fodor et al., 1994; Klumpp et al., 1997). Alternatively, NP might exert such an effect by direct protein–protein interactions with the PB1 and/or PB2 subunits of the RNA polymerase (Biswas et al., 1998; Medcalf et al., 1999; Poole et al., 2004).

Irrespective of the possible mechanisms by which NP may control viral replicative transcription, there is a long-standing hypothesis that its intracellular concentration is important. It is proposed that, at low NP levels, mRNA transcription is favoured, but as translation of these messages proceeds and NP concentration rises, the balance of RNA synthesis tips towards genome replication. Experimental evidence supporting this scheme has been found in non-segmented, negative-strand virus systems (Blumberg et al., 1985; Takeuchi et al., 1987). However, the reasons that NP is required for genome replication remain uncertain. Both v- and cRNA are encapsidated to form functional RNPs (Pons, 1971; Hay et al., 1977b), so this provides part of the explanation (Shapiro & Krug, 1988). Functional analysis of two ts NP mutants that are specifically defective for genome replication supports this hypothesis, because both mutants lose the ability to bind RNA at the non-permissive temperature (Medcalf et al., 1999). However, NP may also play a direct regulatory role in controlling genome replication. Such a role would be particularly likely for positive-strand RNA synthesis, where two types of RNA, differing in their mode of transcription initiation and termination, are made from the same template. One possibility is that newly synthesized NP alters the structure of the RNA promoter sequences found at the termini of each genome segment, thereby biasing the polymerase towards replicative transcription (Hsu et al., 1987; Fodor et al., 1994; Klumpp et al., 1997). Alternatively, NP might exert such an effect by direct protein–protein interactions with the PB1 and/or PB2 subunits of the RNA polymerase (Biswas et al., 1998; Medcalf et al., 1999; Poole et al., 2004).

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**METHODS**

**Cells, virus, plasmids and antibodies.** Human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with l-glutamine, penicillin, streptomycin and 10% fetal calf serum (FCS). Influenza virus strain A/PR/8/34 (PR8) was propagated in 10-day-old embryonated eggs as described previously (Elton et al., 2001). Infections were carried out at an m.o.i. of 5.

Plasmids pcDNA-PB2, -PB1 and -PA were created by ligation of fragments excised from pAPR102, pAPR206 and pAPR303 (Young et al., 1983) with BamHI, HindIII or EcoRI, respectively, into similarly linearized pcDNA3. Construction of plasmid pcDNA-NP is described elsewhere (Carrasco et al., 2004). pPoll(−)NS.CAT.RT, containing an anti-sense chloramphenicol acetyltransferase (CAT) gene flanked by the non-coding sequences of influenza A/WSN/33 virus segment 8, under control of a human RNA polymerase I promoter (Pol-I) and upstream of a hepatitis δ ribozyme sequence, was generously provided by Ervin Fodor (University of Oxford) and is described elsewhere (Pleschka et al., 1996; Fodor et al., 2002). A positive-sense CAT reporter [pPoll(+)-NS.CAT] was constructed similarly by PCR amplification from pPoll(−)NS.CAT.RT using primers PD211 (5’-GCATGCT-TGTAATGCAAAAACAGAGGTAGC-3’) and PD212 (5’-GCATGCGCTTTCCGGCGATGAAACAGGTGTTG-3’). PCR products were digested with SalI and ligated into the vector pPoll.SapLIrib, kindly provided by Ervin Fodor. Plasmids pT7(+)-NS.CAT and pT7(−)-NS.CAT contained positive- and negative-sense CAT gene sequences flanked by non-coding sequences from influenza A virus segment 8, under control of a T7 promoter in a pUC19 background. The sequences were amplified from the appropriate pPol-LNS.CAT segments by PCR using primers PD237 (5’-GGCGACAGTTC-ATACACGACTCAGTATAAGTAGAAACAGGTG-3’) and PD238 (5’-GGCCCTCTAGACGCTACGAGCAGAAGAAAAGAGGGTGAC-3’) for pT7(−)-NS.CAT and PD213 (5’-GGCGACAGTTCAGACATGACCTA-CTTAAGAAACAGACGGTGAC-3’) and PD214 (5’-GGCTCTAGACGCTACGAGCAGAAGAAAAGAGGGTGAC-3’) for pT7(+)NS.CAT. The products were digested by HindIII and Xbal and cloned into similarly digested pUC19. Digestion of these plasmids with Hpal allowed T7 in vitro transcription of an RNA with the correct 3’ and 5’ termini.

**Influenza virus gene-expression assay.** 1 × 10⁶ cells per 35 mm well in 1 ml complete medium were transfected in suspension with plasmid DNA by using cationic liposomes (Lipofectin; Gibco-BRL). To reconstitute RNPs, 0-25 µg each of pcDNA-PB1, -PB2 and -PA and 50 ng of either pPol-I(+)NS.CAT or pPol-I(−)NS.CAT were transfected with varying amounts of pcDNA-NP. The total amount of DNA in each transfection mix was normalized by the addition of pcDNA3 vector. Optimal amounts of plasmids other than pcDNA-NP were established by separate titration experiments (data not shown). Following incubation at 37°C for 3 days, CAT accumulation was quantified by a commercial ELISA (Roche Diagnostics).

**Primer-extension analysis of viral RNA.** Total cellular RNA was isolated by using a commercial kit (SV Total RNA Isolation System; Promega) according to the manufacturer’s instructions. After spectrophotometric quantification and normalization, RNA (1-5 µg) was reverse-transcribed by using avian myeloblastosis virus reverse transcriptase (Promega) and 0-4 pmol each of the appropriate DNA oligonucleotides that had been 5’ end-labelled with [α-32P]ATP. Following 30 min incubation at 42°C, samples were heated to 90°C for 10 min and separated by 6% urea-PAGE. Radiolabelled products were detected by autoradiography and quantified by densitometry using NIH Image software (http://rsb.info.nih.gov/nih-image/). The oligonucleotides used were 5’-GGGATCTACGTTGATCGTTGAC-3’ (for detection of vRNA-sense CAT transcripts), 5’-ATGTTCTTATGATGGGGG-3’ (positive-sense CAT transcripts), 5’-GTCCTAGCTCATCCGAGATC-3’ (positive-sense segment 5'), 5’-GTCCTATTTTTGCGCATGC-3’ (negative-sense CAT transcript), 5’-GACCATATTCTCCGCAAGCATG-3’ (negative-sense segment 3’).
5'-GTCTTCGAGCTCTCGGAG-3' (segment 5 vRNA), 5'-TCAA-GTCTCGGTCGGATCTCG-3' (positive-sense segment 7) and 5'-ACCGTCGCTTTAAATACGG-3' (segment 7 vRNA). The predicted size of primer-extension product for each oligonucleotide was validated by comparison with matching DNA sequencing ladders (CAT; see Results section, segments 5 and 7; data not shown). Primers were designed so that the reverse-transcription products from m-, c- and vRNA were distinguishable by gel electrophoresis. Preliminary experiments established that simultaneous detection of positive- and negative-sense RNA species gave equivalent results to separate reactions carried out with individual primers (data not shown).

**Protein analyses.** For Western blots, cell lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose. Blots were probed with primary followed by secondary antibodies conjugated to horse-radish peroxidase (DAKO) and developed by chemiluminescence (ECL reagent; Amersham Biosciences). For immunofluorescence, subconfluent cells on glass coverslips were fixed with PBS containing 4% formaldehyde for 20 min and washed in PBS containing 2% FCS. Cells were permeabilized with 0.2% Triton X-100 before incubation with primary antibodies, followed by fluorophore-conjugated secondary antibodies in PBS/FCS. Coverslips were mounted in Citifluor (Agar Scientific) and examined under a Leica TCS NT confocal microscope.

**RESULTS**

**Effect of NP concentration on RNA synthesis by recombinant RNPs**

To test the hypothesis that an increase in NP expression causes a switch from genome transcription to replication, we first utilized a recombinant system that recreates functional influenza virus RNPs by transfecting cells with plasmids that separately express the three polymerase proteins, NP and a model v- or cRNA segment containing a CAT gene (Huang et al., 1990; Fodor et al., 2002). 293T cells were transfected with plasmid mixtures containing increasing amounts of the pcDNA-NP plasmid and constant amounts of pcDNA-PB1, -PB2 and -PA and plasmids that expressed CAT v- or cRNA. After 3 days, cell lysates were analysed by Western blotting with anti-NP serum to determine whether the amount of NP expressed in the cells was proportional to the amount of transfected pcDNA-NP. No reactivity was detected in samples from cells that were not transfected with pcDNA-NP (Fig. 1a, lanes 7). Otherwise, a polypeptide with the same electrophoretic mobility as NP from purified virus was detected (at the exposure shown) from all cells transfected with >111 ng pcDNA-NP (Fig. 1a, lanes 4–6, 8). NP accumulation increased in proportion to the dose of plasmid and was essentially the same whether the genome segment supplied was cRNA- or vRNA-sense (Fig. 1a). Furthermore, the expression levels of other RNP components were largely unaffected by co-expression of increasing amounts of NP, except at the very highest doses (3 μg) of pcDNA-NP (PB2, Fig. 1b; PB1, data not shown). We also compared the amounts of NP expressed in the recombinant system with that of authentic virus infection, finding that NP was detected in similar amounts in lysates from cells transfected with 250 ng pcDNA-NP and from infected cells harvested between 4 and 8 h post-infection (p.i.) (Fig. 1c, compare lane 3 with lanes 5–7). Therefore, the recombinant system for reconstituting RNPs in cells permits the expression of a wide range of NP concentrations that, in the middle of the range, are comparable to those found in infected cells.

Next, we examined the effect of NP levels on virus-directed gene expression in the transfection system by quantifying CAT accumulation as a marker of mRNA transcription. CAT expression was detected above the background level that was seen in the absence of NP even with the lowest...
dose of NP plasmid, irrespective of whether the model genome segment supplied was positive-sense (therefore requiring genome replication before mRNA transcription) or negative-sense (allowing direct transcription) (Fig. 2a). Transfection of higher amounts of pcDNA-NP initially caused a steep rise in CAT accumulation, which reached a plateau with plasmid doses of around 0.3–1.0 μg per transfection for reactions primed with either v- or cRNA molecules. Further increases in the amount of NP plasmid transfected caused a decrease in viral gene expression with input model viral genomes of both senses (Fig. 2a).

The hypothesis that increased levels of NP promote genome replication suggests that the decrease in virus gene expression observed at high concentrations of NP could have resulted from an increase in c- and/or vRNA synthesis, at the expense of transcription. To test this, we first validated an assay to examine virus-specific RNA synthesis directly. Total RNA was extracted from cells transfected with plasmids expressing the vRNA-sense CAT segment and the three P proteins, but with or without pcDNA-NP. Positive-sense CAT RNA was then analysed by a reverse transcriptase primer-extension assay. No products were observed from cells that were not transfected with the NP plasmid (Fig. 3a, lane 7), but two classes of primer-extended transcripts were seen from RNA from cells expressing all four RNP polypeptides. These were a smaller unique species and a larger heterogeneous family of at least four or five products, differing by 1 nt (Fig. 3a, lane 4). The unique transcript co-migrated with a primer-extension product that was produced from a synthetic cRNA molecule transcribed in vitro from plasmid pT7(+NS.CAT (compare lanes 1 and 4). Furthermore, reference to a DNA sequencing ladder derived from pT7(+NS.CAT using the same oligonucleotide as the primer-extension reaction (lanes 4 and T, G, C, A) showed that the smaller product was the predicted size for the 5’ end of cRNA. The primer-extension products were seen to be some 9–13 nt longer than the shorter species, consistent with the presence of host-cell pre-mRNA-derived, 5’-capped RNA extensions (Plotch et al., 1981). Thus, the 5’ ends of the two classes of positive-sense CAT RNA detected by this system are consistent with their formation from cRNA and mRNA.

To test this further, we examined the 3’ ends of the transcripts by poly(A) selection. Again, no products were detected from RNA from cells lacking an NP gene (Fig. 3a, lanes 5 and 6). Of the CAT-specific transcripts detected from cells containing functional viral RNPs, the larger products were mostly present in the poly(A) fraction (lane 2), whereas the smaller species was not polyadenylated (lane 3), confirming their identities as mRNA- and cRNA-derived products, respectively. Densitometric quantification of replicate experiments indicated that the cRNA-derived species represented approximately 5–10% of the total positive-sense RNA (data not shown), in agreement with values measured for infected cells (Hay et al., 1977b; Barrett et al., 1979; Herz et al., 1981). Similar primer-extension analyses carried out with a primer complementary to negative-sense CAT RNA confirmed its ability to detect vRNA (data not shown, but see Fig. 3b).

![Fig. 2. Quantitative analysis of the effect of NP titration on viral gene expression and c- and vRNA synthesis. Cells were transfected with plasmids expressing the three P proteins, a model cRNA (○) or vRNA (■) segment containing a CAT gene and the plotted amounts of pcDNA-NP. Samples were harvested at 72 h post-transfection. (a) CAT polypeptide accumulation was quantified by ELISA and calculated as a percentage of the maximum value after subtraction of the background seen in the absence of NP. Means ± SD of three (cRNA) or four (vRNA) experiments are plotted. (b, c) CAT-specific RNA extracted from parallel transfections was detected by radiolabelled primer extension and urea-PAGE (see Fig. 3) and quantified (in arbitrary units) by densitometry of exposed X-ray film. Ratios of cRNA (b) and vRNA (c) to mRNA for each amount of transfected pcDNA-NP are plotted.](image-url)
Next, we examined the viral RNA species synthesized by recombinant RNPs across a range of NP concentrations, using both oligonucleotides to simultaneously detect m-, c- and vRNA. When NP was omitted from the transfection mix, the only detectable product was vRNA in the case of cells transfected with pPol-I(2-NS.CAT, or cRNA for cells transfected with pPol-I(+NS.CAT (Fig. 3b, lanes 2 and 9, respectively), probably representing detection of Pol-I-transcribed virus-like RNA from the input plasmids. However, transfection of even the smallest amount of NP plasmid tested resulted in viral transcription and replication, producing detectable c-, v- and mRNA (Fig. 3b, lanes 3 and 10). In broad agreement with the data obtained for CAT polypeptide accumulation, further increases in the amount of transfected NP plasmid quickly led to maximal levels of mRNA synthesis that then reached a plateau or declined slightly with the highest doses of NP (Fig. 3b, lanes 4–8 and 11–15). Similarly, maximal amounts of v- and cRNA were produced at relatively low doses of NP plasmid (37 ng; Fig. 3b, lanes 3 and 10). However, synthesis of v- and cRNA then declined with higher doses of NP, to barely detectable levels at the highest concentration of plasmid tested (3 μg; Fig. 3b, lanes 8 and 15). Overall, visual inspection of the data did not favour the hypothesis that increasing concentrations of NP are associated with higher levels of genome replication; indeed, the converse appeared to be true. Confirming this, quantification of RNA levels by densitometry and plotting the ratios of cRNA to mRNA against amount of transfected pcDNA-NP revealed a shift towards synthesis of mRNA, with an overall fourfold decrease in the ratio of cRNA to mRNA with increased pcDNA-NP, regardless of polarity of the input viral segment (Fig. 2b). The ratio of vRNA to mRNA reflected a similar fivefold trend in favour of transcription with increasing amounts of NP, again irrespective of the polarity of model viral segment introduced. The change in ratio of replicative to transcriptional products was progressive across the entire NP titration and did not occur solely at the highest doses of NP plasmid. This argues for a direct effect of increased NP concentration, rather than an indirect effect of decreased polymerase concentration. Nevertheless, it is possible that decreased expression of the polymerase at very high levels of NP also contributes to decreased genome replication. Quantification of three replicate experiments showed the same magnitude decrease in replicative synthesis compared with transcription with increased amounts of NP plasmid (data not shown). Therefore, in a recombinant setting, increased expression of NP does not promote viral genome replication, but instead leads to a transcriptional bias.

It has been demonstrated previously that, when NP is expressed by itself, higher levels of expression bias it towards cytoplasmic accumulation (Digard et al., 1999; Elton et al., 2001). To determine whether the same trend was apparent in cells producing functional RNPs, parallel samples from each transfection were examined by indirect immunofluorescence for NP and Nup62, a component of the nuclear pore complex. No NP staining was detected in control cells [Fig. 4a(i)]. When pcDNA-NP-transfected
cells were examined 1 day post-transfection, the majority of NP was found in the nucleus at all plasmid doses tested (data not shown). At 3 days post-transfection, cells transfected with the lowest dose of NP plasmid (11 ng) showed a mixture of nuclear and cytoplasmic staining [Fig. 4a(ii)]. When replicate experiments were stained and scored for NP localization, it was found that, on average, approximately half of the transfected cells still contained nuclear NP (Fig. 4b). As the amount of transfected plasmid was raised, fewer cells contained nuclear NP until, at the very highest dose, the protein was almost exclusively cytoplasmic [Fig. 4a(iii and iv) and b]. Similar results were obtained when experiments were carried out by using a plasmid expressing CAT cRNA (data not shown). Therefore, NP exhibits the same dose-dependent localization pattern when expressed alone or with vRNA, PB1, PA and PB2: a shift from nuclear to cytoplasmic localization with increased NP expression. In light of the preceding experiments, this suggests that a reduction in synthesis of replicative RNA types may correlate with increased or earlier cytoplasmic localization of NP.

**Effect of prior NP expression on viral RNA synthesis**

Next, we tested whether increased levels of NP influenced genome replication in the context of virus infection. Cells were transfected with varying amounts of pcDNA-NP as before, incubated overnight to establish a concentration gradient of the protein and then superinfected with influenza virus and viral RNA synthesis was analysed. Pilot experiments where cells were doubly transfected with plasmids expressing NP and green fluorescent protein, infected with virus and viral gene expression was monitored by immunofluorescent staining for NS1 confirmed that prior expression of NP in a cell did not inhibit subsequent influenza virus infection (data not shown). In addition, because RNA synthesis in infected cells follows a regulated pattern in which the three classes of transcript are made at distinct times (Hay et al., 1977b; Barrett et al., 1979; Smith & Hay, 1982; Shapiro et al., 1987), we first examined normal viral RNA synthesis in our system to establish the optimal time point at which to analyse the effect of supplying extra NP. When the RNA products from segment 7 (encoding M1 and M2; representative of the late class of influenza virus genes) were analysed, mRNA accumulation peaked at 4 h p.i. and then declined (Fig. 5a, lane 2). In this particular experiment, cRNA accumulation also reached a maximum at 4 h p.i., although, in other experiments, there was little difference visible between its levels at 2-5 and 4 h p.i. (data not shown). In contrast, vRNA accumulation increased steadily throughout the time-course, reaching a maximum at 4 h p.i., although, in other experiments, there was little difference visible between its levels at 2-5 and 4 h p.i. (data not shown). In contrast, vRNA accumulation increased steadily throughout the time-course, reaching a maximum at 8 h p.i. (Fig. 5a, lane 4). No significant primer-extension products were observed from RNA taken from mock-infected cells (Fig. 5a, lane 5). Similar kinetics (with the time points used here) were observed for the RNA products of segment 5, encoding NP, a representative early gene (data not shown). Thus, the virus–cell combination used here behaves similarly to that expected from previous analyses of viral RNA synthesis (Hay et al., 1977b; Barrett et al., 1979; Smith & Hay, 1982; Shapiro et al., 1987).
Therefore, we transfected cells with varying amounts of pcDNA-NP and superinfected them with influenza virus after overnight incubation to allow expression of NP. RNA was isolated at 6 h p.i. (when, ordinarily, vRNA accumulation would be submaximal) and analysed by primer extension. One minor background primer-extension product was seen after reverse transcription of RNA from mock-infected cells using oligonucleotides specific for segment 7, whereas m-, c- and vRNA species were all detected from infected, but untransfected cells (Fig. 5b, lanes 7 and 8). The amounts of these products were not altered substantially by mock transfection of the cells (lane 1), nor was any clear trend observable following transfection of increasing amounts of pcDNA-NP (lanes 2–6). When segment 5-specific RNAs were analysed, a primer-extension product that presumably resulted from cross-hybridization with a cellular RNA was observed from all samples, independent of their prior transfection or infection (Fig. 5c). The only novel primer-extension product observed from pcDNA-NP transfected, but mock-infected cells corresponded to that predicted for plasmid-derived NP mRNA (Fig. 5c, lane 8), whereas RNA from infected, but mock-transfected cells gave products that were diagnostic of v-, c-, and mRNA (lane 1). Transfection of increasing amounts of pcDNA-NP led to the detection of increasing amounts of the plasmid-derived mRNA species, confirming successful expression of exogenous NP mRNA in the cells (Fig. 5c, lanes 2–7). However, no significant alteration in the accumulation of virus-transcribed RNA species was seen. Densitometric quantification of the ratios of the replicative RNA species to mRNA from replicate experiments confirmed that the relative amount of cRNA from segments 5 and 7 was unaltered by prior expression of NP in the cell (Fig. 6). The ratio of segment 5 vRNA to mRNA was similarly insensitive to pcDNA-NP transfection, whereas that of segment 7 vRNA showed an overall slight decrease (of around one-third; statistically significant at the level of $P<0.02$, as assessed by Student’s t-test, for plasmid doses of 111–1000 ng) after transfection of even the smallest amount of NP plasmid (Fig. 6). However, no further decrease in the ratio of vRNA to mRNA was seen with increased expression of NP. In addition, no significant changes to viral RNA synthesis were seen when the accumulation of segment 5 or 7 species was examined at 4 h p.i. (data not shown). Overall, we find no evidence that increased levels of NP promote the synthesis of replicative RNA products in infected cells.

**DISCUSSION**

A hallmark of the replication cycle of negative-sense RNA viruses is the synthesis of shorter than unit-length mRNAs from a genomic RNP. Genomes are replicated via a full-length, positive-sense antigenome strand that also forms an RNP. In all cases analysed, the encapsidating NP is an essential component of this process. The hypothesis, first formulated for a non-segmented virus (Leppert et al., 1979; Blumberg et al., 1981; Arnheiter et al., 1985), that increasing
concentrations of this NP serve to switch polymerase activity from transcription to replication has been considered to be plausible in all systems. However, where this hypothesis has been tested directly, it has not been supported. When the N or N and P proteins of respiratory syncytial virus were titrated into a transfection-based minigenome replication system, no bias towards genome replication was detected by radiolabelled primer extension and urea-PAGE (see Fig. 5) and quantified (in arbitrary units) by densitometry of exposed X-ray film. Mean ratios ± SD from three independent experiments of cRNA (circles) and vRNA (squares) to mRNA for each amount of transfected pcDNA-NP are plotted. Asterisks indicate values that are significantly different (Student's t-test, \( P < 0.02 \)) from the untreated sample.

**Fig. 6.** Quantitative analysis of the effect of prior NP expression on viral RNA synthesis. Segment 5 (filled symbols) and segment 7 (open symbols)-specific RNA extracted from cells transfected with the indicated amount of pcDNA-NP and super-infected with virus was detected by radiolabelled primer extension and urea-PAGE (see Fig. 5) and quantified (in arbitrary units) by densitometry of exposed X-ray film. Mean ratios ± SD from three independent experiments of cRNA (circles) and vRNA (squares) to mRNA for each amount of transfected pcDNA-NP are plotted. Asterisks indicate values that are significantly different (Student's t-test, \( P < 0.02 \)) from the untreated sample.

Increases in NP expression (at levels still well below that encountered in infected cells) did indeed promote increased accumulation of c- and vRNA, but also of mRNA. However, further increases in NP expression inhibited genome and antigenome accumulation. Furthermore, when the ratio of these replicative species to viral mRNA was considered, a consistently negative correlation between the amount of NP and the relative amount of genome replication compared with transcription was seen, even for the lowest concentrations of NP.

Although plasmid-based systems for reconstituting RNPs have been used widely to study influenza virus RNA synthesis, they probably do not reproduce all facets of authentic virus transcription, particularly with regard to its temporal regulation. During virus infection, cRNA is only synthesized at early times p.i., following which vRNA and mRNA amplification occurs. Whilst vRNA synthesis continues later in infection, mRNA transcription peaks and then declines (Fig. 5a; Hay et al., 1977b; Barrett et al., 1979; Smith & Hay, 1982; Shapiro et al., 1987). In contrast, time-course experiments analysing viral RNA synthesis in the recombinant system used here show no evidence of any such synthetic cascade, with the amounts of all three RNA species simply increasing over time (data not shown). However, like authentic virus infection, cRNA remains a relatively low-abundance species. As such, the system can be considered to be a partially deregulated model of viral transcription, which perhaps lessens the confidence that we can place in our finding that increased concentrations of NP do not stimulate genome replication. However, consistent results were obtained from infected cells that were manipulated to contain varying amounts of NP prior to virus inoculation. No significant alteration was seen in the overall amounts of cRNA for segments 5 and 7, or segment 5 vRNA. Prior expression of NP did cause a slight alteration in the ratio of segment 7 vRNA to mRNA but, as with the recombinant CAT segment, this was a decrease, rather than an increase. The infection system relies on transfection of the cells with an NP-expressing plasmid before subsequent virus infection. Although virtually every cell would be expected to be infected, the same would not be true of transfection, leading to a background of virus transcription taking place in the absence of extra NP. Accordingly, we monitored transfection efficiencies by immunofluorescence to establish the size of this background, finding that approximately one-third of cells were transfected successfully (data not shown). Statistical analysis of the densitometry data suggests that an overall twofold change in the ratio of genome or antigenomic RNAs to mRNA would be detected reliably, because the less than twofold change in ratio of segment 7 vRNA to mRNA was partially statistically significant. Therefore, for a constant amount of mRNA, a fourfold change in v- or cRNA accumulation would be required in the transfected cells to achieve an overall twofold increase in the ratio of genomic or antigenomic RNA species to mRNA. As the only change seen was a minor decrease in the relative amount of segment 7 vRNA, we can exclude...
any major (greater than fourfold) stimulatory effect of increased NP expression on virus genome replication.

If the relative rate of genome replication is not directly proportional to the intracellular concentration of NP, then what explains the clear necessity for NP for replicative RNA synthesis? One model is that other factors (e.g., the polymerase and/or cellular factors) regulate RNA synthesis, keeping mRNA as the predominant positive-sense RNA. NP is nonetheless an essential cofactor, because of a requirement for co-transcriptional encapsidation of nascent c- and vRNA segments. This model is consistent with the heterogeneous size of short vRNA products that are synthesized by infected-cell extracts depleted of NP (Shapiro & Krug, 1988) and the finding that ts NP mutants that are specifically defective for replicative transcription lose RNA-binding activity at the elevated temperature (Medcalf et al., 1999). It is also consistent with the observation that NP is not required in vitro for unprimed transcription initiation (Lee et al., 2002). Indeed, these findings and our current data are consistent with a recent study (published while this manuscript was under review) that suggests that there is no switch mechanism regulating cRNA synthesis and that the requirement for protein synthesis during genome replication reflects degradation of ‘naked’ cRNA that is not protected by encapsidation into an RNP (Vreede et al., 2004). On the other hand, identification of NP mutations that affect v- and cRNA synthesis differentially is highly suggestive of a direct regulatory role for the protein (Thierry & Danos, 1982; Markushin & Ghendon, 1984; Mena et al., 1999). This regulation could be exerted through NP–polymerase interactions (Biswas et al., 1998; Mena et al., 1999; Medcalf et al., 1999; Poole et al., 2004) or through NP-mediated modification of the panhandle promoter structure (Fodor et al., 1994; Klumpp et al., 1997; Lee et al., 2003). To be consistent with the findings reported here, one would have to postulate that only small amounts of NP are required to enable replicative synthesis and that some other factor prevents cRNA synthesis from rising above 5–10% of positive-sense transcription.

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