Assignment of mutant tsN19 (complementation group E) of respiratory syncytial virus to the P protein gene

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The mutation responsible for the temperature-sensitive (ts) phenotype of mutant tsN19 (complementation group E) of respiratory syncytial virus has been located to the P protein gene. Viral protein synthesis was completely restricted at 39 °C, and the tsN19 P protein did not react with an anti-P monoclonal antibody (MAb) (3-5) at 33 °C. Reversion of temperature sensitivity restored reactivity with MAb 3-5. Nucleotide sequence determination and in vitro expression of cDNA clones of P mRNA derived from wild-type, tsN19 and non-ts revertant-infected cells, revealed that temperature sensitivity and loss of reactivity with MAb 3-5 were consequences of a Gly → Ser amino acid change at position 172. A low Mr polypeptide, which represented the C-terminal 93 amino acids of the P protein, was produced by internal initiation in the P open reading frame during in vitro translation, and a similar product was detected transiently in vivo.

Introduction

The genome of respiratory syncytial (RS) virus comprises 10 genes ordered in a linear sequence from a 3'–terminal promoter with a single 68 nucleotide overlap of the end of the penultimate 22K protein gene and the start of the 5’–proximal L protein gene (Huang & Wertz, 1982; Collins & Wertz, 1983; Dickens et al., 1984; Collins et al., 1986, 1987). Eight complementation groups of temperature-sensitive (ts) mutants have been described which are presumed to correspond to eight of these 10 transcriptional units (Wright et al., 1973; Gimenez & Pringle, 1978; Pringle et al., 1981). Assignment of these complementation groups will assist analysis of gene function. Previously we have presented evidence for assignment of complementation groups B and D to the attachment (G) and matrix (M) protein genes respectively (Caravokyri & Pringle, 1991). Here we describe evidence for assignment of a third complementation group.

Methods

Viruses and cells. The A2 wild-type strain (subgroup A) was obtained originally from Dr R. M. Chanock. The origin of the RSN-2 wild-type strain (subgroup B) and mutant tsN19 has been described previously (Faulkner et al., 1976). The mutant, wild-type and non-ts revertant viruses were propagated in BS-C-1 cells maintained in the Glasgow modification of Eagle's medium supplemented with 2% foetal calf serum and antibiotics. Infectivity assays were carried out in CO2 incubators. Radiolabelling experiments were carried out in small flasks totally immersed in a precision water bath. The permissive temperature was 33 °C and the restrictive temperature 39 °C. Non-ts revertants were isolated by propagation of virus from the few plaques which appeared on tsN19-inoculated plates after incubation for 5 to 7 days at 39 °C.

Monoclonal (MAb) and polyclonal antibodies. The polyclonal bovine anti-RS virus serum was provided by Dr Geraldine Taylor (Institute of Animal Health, Compton, U.K.) and had been prepared by immunization of a gnotobiotic calf. The anti-P protein MAbs 8268, 9178 and 9516, and the anti-N MAbs N2 and N7 were obtained from Dr C. Örvell (Karolinska Institute, Stockholm, Sweden), and the anti-P MAbs 3-5 and 4-14 from Dr Beatriz Gimenez (University of Aberdeen, U.K.).

Radiolabelling, immunoprecipitation and SDS-PAGE. Standard methods were employed (Caravokyri & Pringle, 1991).

cDNA cloning and nucleotide sequencing. cDNA clones of P mRNA were produced by reverse transcription followed by polymerase chain reaction (PCR) amplification and were sequenced by the dideoxynucleotide chain termination method (Caravokyri & Pringle, 1991).

In vitro protein synthesis. Total cytoplasmic RNA from infected or uninfected cells (up to 10 μg) or uncapped RNA transcripts produced in vitro (150 to 300 ng) were translated in a nuclease-treated rabbit reticulocyte lysate (NE-N) as previously described (Caravokyri & Pringle, 1991, 1992).

Results

Viral protein synthesis

Previously, cells infected by mutant tsN19 (RSN-2 strain) were found to be negative for both intracellular and surface immunofluorescent staining (Pringle et al., 1981), suggesting that the tsN19 defect was exhibited
early in infection. This result is confirmed by the experiment illustrated in Fig. 1. BS-C-1 cell cultures were infected with mutant tsN19, a non-ts revertant or wild-type virus, and incubated at permissive (33 °C) or restrictive (39 °C) temperatures and radiolabelled with \([^{35}S]\)methionine as described previously (Caravokyri & Pringle, 1991). No intracellular or virion-associated polypeptides could be detected in tsN19-infected cultures incubated at 39 °C (Fig. 1 a and b, respectively). In contrast, non-ts revertants isolated from tsN19 stocks exhibited efficient viral protein synthesis at 39 °C and their profiles were indistinguishable from that of the RSN-2 wild-type virus.

The P protein of mutant tsN19 did not react with anti-P MAb 3-5 after infection and radiolabelling at the permissive temperature of 33 °C (Fig. 1 c). This negative
reaction has also been observed by Western blotting and by immunofluorescence (B. Gimenez, personal communication). The tsN19 P protein was found to react with all other anti-P MAbs tested (two of which are shown in Fig. 1c), including MAbs representing the three B subgroup epitopes defined by Orvell et al. (1987). The P protein of revertant clone R3/6 (Fig. 1c) and of another five independently isolated revertants (not shown) was immunoprecipitated by anti-P MAb 3-5. The simultaneous reversion of the ts phenotype and restoration of reactivity of the P protein with MAb 3-5 in the non-ts revertants indicated that the P protein of mutant tsN19 was the site of its ts lesion.

The intracellular stability at 39 °C of the P protein of tsN19 presynthesized at 33 °C was examined by pulse-radiolabelling at 33 °C followed by a chase period at 39 °C. Fig. 2 shows the negative profile of tsN19-infected cultures incubated at 39 °C immediately post-infection (lane 1). However, when tsN19-infected cells that had been maintained and labelled at 33 °C were chased at 39 °C for up to 2 h, no decrease of the P protein band was observed. In another experiment BS-C-1 cells were infected with wild-type virus, mutant tsN19 or mutant tsN1 which expresses a thermostable M protein (Caravokyri & Pringle, 1991), and shifted up to 39 °C after 24 h incubation at 33 °C. The yields of infectious virus from the tsN19-infected cultures after 24 h at 39 °C were similar to those from wild-type virus-infected cultures, whereas the yields from tsN1-infected cultures showed no enhancement over the yields obtained from replicate cultures maintained at 33 °C throughout (data not shown). These results established that the tsN19 protein presynthesized at 33 °C was not thermolabile and that it retained its functional activity at 39 °C.

Production and sequencing of P cDNA clones

In order to identify the site of the tsN19 lesion at the nucleotide level, cDNA clones of the P mRNA were produced as described elsewhere (Caravokyri & Pringle, 1992). Two different clones from each type of viral mRNA (i.e. wild-type, non-ts revertant or tsN19), representing the two opposite orientations of insertion into the plasmid multiple cloning site, were selected for sequence determination. The nucleotide differences found in each clone relative to the consensus RSN-2 sequence (GenBank accession number M67450; Caravokyri & Pringle, 1992) are listed in Table 1. These differences could be artefacts of the in vitro synthetic process and/or due to variation in the original mRNA population. Indeed, the greater nucleotide variability seen in the two tsN19-derived clones, compared with the revertant clones which were derived from more recently plaque-purified virus, may indicate greater mRNA heterogeneity of the mutant stock. However, of the three or four substitutions identified in the two clones from mutant tsN19, only the G → A substitution at position 531 was common to both clones. Furthermore this substitution caused the only predicted amino acid change in one of the clones (N23), indicating that this is the site of the tsN19 mutation.

In vitro synthesis of the P protein

To confirm that the Gly → Ser change (predicted from the sequence of the two mutant clones) correlated with the loss of the epitope defined by MAb 3-5, it was necessary to use this antibody for immunoprecipitation of P proteins expressed from the PCR-derived clones. Therefore the P cDNA clones were transcribed in vitro and the P mRNA-sense transcripts were used to programme protein synthesis in the rabbit reticulocyte lysate system. Both the tsN19-derived cDNA clones produced a P-specific protein which comigrated with the in vitro translated tsN19 P protein (Fig. 3). Also both the P proteins from revertant clone R34 (Ile → Val) and the mutant clone N23 (Gly → Ser) were immunoprecipitated with anti-P MAb 8268. However, in contrast to the R34 P protein, the N23 P protein which contained the single

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**Fig. 2. Intracellular stability of the tsN19 P protein.** Replicate BS-C-1 cell monolayers were infected with tsN19 and incubated at 33 °C or 39 °C for 48 h, then pulse-labelled with [35S]methionine for 10 min. One culture from each temperature was lysed and viral proteins were immunoprecipitated with polyclonal antiserum immediately after removal of the radiolabel (lanes 1, 39 °C and lane 2, 33 °C). The remaining cultures were similarly treated after further incubation in medium containing non-radioactive methionine for periods up to 120 min (10, 20, 30, 45, 60 and 120 min; lanes 3 to 8, respectively). One set of cultures was shifted from 33 °C to 39 °C after pulse labelling (lanes 3 to 8). Control cultures were maintained throughout at 33 °C (lane 3) or 39 °C (lane 9). M, markers are shown in lane M. Pbrk is a breakdown product of the P protein.
Table 1. Nucleotide differences of individual P cDNA clones and predicted changes in their respective proteins

<table>
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<tr>
<th>cDNA clone</th>
<th>Virus origin</th>
<th>Nucleotide position</th>
<th>Substitution</th>
<th>Amino acid position</th>
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<tr>
<td>N19 tsN19</td>
<td></td>
<td>297</td>
<td>A→G</td>
<td>94</td>
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<td></td>
<td></td>
<td>318</td>
<td>T→C</td>
<td>101</td>
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<td></td>
<td></td>
<td>531</td>
<td>G→A</td>
<td>172</td>
<td>Gly→Ser</td>
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<td></td>
<td></td>
<td>592</td>
<td>T→C</td>
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<td>152</td>
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<tr>
<td></td>
<td></td>
<td>531</td>
<td>G→A</td>
<td>172</td>
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<td></td>
<td></td>
<td>647</td>
<td>C→G</td>
<td>270</td>
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<tr>
<td>R29 ts R3/6 (non-ts revertant of tsN19)</td>
<td></td>
<td>224</td>
<td>T→C</td>
<td>69</td>
<td>None</td>
</tr>
<tr>
<td>R34 ts R3/6 (non-ts revertant of tsN19)</td>
<td></td>
<td>513</td>
<td>A→G</td>
<td>166</td>
<td>Ile→Val</td>
</tr>
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</table>

Gly → Ser change did not react with MAb 3-5 (data not shown). Therefore, the Gly → Ser change at position 172 in the P protein of clone N23 seems to be solely responsible for the negative reaction to anti-P MAb 3-5. Since the same phenotype was observed in vivo, and recovery of the 3-5 epitope was coordinate with restoration of the non-ts phenotype in the revertants, the Gly → Ser change at amino acid position 172 of the P protein is the likely site of the ts lesion in mutant tsN19.

Internal initiation of translation in the P open reading frame (ORF)

Among the in vitro translation products of RNA transcripts produced from the P cDNA clones, a low Mr band of approximately 15K to 16K was invariably detected. Although additional (fainter) bands in the profiles could be correlated with similar bands present in in vitro translations of P mRNA from infected cells (probably reflecting some proteolytic degradation), only trace amounts of this low Mr band could be detected by in vitro translation of P mRNA synthesized in vivo (Fig. 3). This small polypeptide designated Pint was also translated from RNA produced by T7 in vitro transcription of a truncated A2 strain P cDNA insert in a different vector (pTZ19; Pharmacia). This cDNA clone (obtained from Dr G. Wertz) contained the full 3' non-coding P mRNA region, but was lacking the first 231 nucleotides of the P mRNA, including the coding sequence for the N-terminal 72 amino acids. The pTZ19/A2-P clone which produced P mRNA-sense transcripts from the T7 promoter could also be translated in vitro into the products shown in Fig. 4a, despite the absence of the authentic P start codon. As shown in Fig. 4(b) the initiator AUG is provided by the SphI site upstream from the PstI site of insertion. The resulting polypeptide is 171 amino acids in length (predicted Mr of 19.3K) and contains two amino acid differences from the authentic P protein. Two major P-specific bands (recognized by polyclonal serum and anti-P MAb 3-5; Fig. 4a), with apparent mobilities of 21K and 23K, were observed. These probably represent the N-terminally truncated forms of the P protein of the A2 wild-type, which migrates as a doublet band (Fig. 5a and c). Two faster migrating, fainter bands (possibly proteolytic fragments) are also present in both the polyclonal and anti-P MAb profiles (indicated by arrows); however, the abundant low Mr polypeptide was not immunoprecipitated by anti-P MAb 3-5. A fainter band of similar mobility to the low Mr polypeptide can be observed also in the A2 strain profile, suggesting that this product is not an artefact of the in vitro expression of the P protein from cloned
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(a) The in vitro translation products of cytoplasmic RNA synthesized in mock-infected cells (lane 1), A2 wild-type-infected cells (lane 2), and of the transcripts synthesized in vitro from recombinant vectors pTZ18/P (− sense; lanes 4 and 5) and pTZ19/P (+ sense; lanes 3 and 6). Recombinant plasmid pTZ18/P produces T7 transcripts of the truncated P cDNA in the antisense orientation. The translation products were immunoprecipitated with polyclonal antiserum (lanes 1 to 4) or MAb 3-5 (lanes 5 and 6). The N-terminally truncated form of the A2 strain doublet is indicated [(A5')P]. Pint is the internally initiated product from the P ORF. The size and relative abundance of this product suggested either that significant proteolytic cleavage occurred at a specific site of the P protein in the absence of other viral proteins, or that translation from an internal start codon occurred in the same ORF as that of the P protein, since this product reacted with anti-P MAb 8268 (Fig. 5a).

(b) The sequence at the site of ligation of the 5'-end of the truncated P cDNA clone with the PstI site of plasmid pTZ19. In vitro transcription of the recombinant plasmid pTZ19/P from the adjacent T7 promoter produced P mRNA sense transcripts (having the same sequence shown here as DNA). The in-frame start codon is provided by the sequence of the immediately upstream SphI restriction site. The numbers in parentheses denote amino acid positions in the intact A2 strain P protein. Residues 71 and 72 are replaced by Met and Pro in the truncated protein.
Fig. 5. Internal initiation in the A2 strain P ORF. (a) In vitro translation products of cytoplasmic RNA extracted from A2-infected cells (lanes 2, 5, 7 and 9), or in vitro synthesized RNA transcripts of the 5' end truncated p cDNA clone (lanes 1, 3, 4, 6 and 8) were immunoprecipitated with polyclonal anti-RS virus serum (lanes 1 and 2) or anti-P MAb 3-5 (lane 3), 8268 (lanes 4 and 5) and 9516 (lanes 6 and 7), or the anti-N MAb N2 (lanes 8 and 9), and analysed by 12.5% SDS-PAGE. The arrow indicates the position of the low Mr polypeptide which reacts with MAb 8268. Mr markers are shown (lane M). (b) A fragment of the P cDNA clone, containing an AUG codon approximately 80 bp from its 5' end (residue 148 of the complete P protein), was subcloned into the multiple cloning site (MCS) of vector pGEM1 which is flanked by the SP6 and T7 promoters (the arrows indicate the direction of transcription). The MCS region between the PstI and BamHI sites replaced by the cDNA insert in the recombinant plasmid is shown as a dashed line. The position of the internal AUG is marked by the arrowhead on the P mRNA sense T7 transcript. (c) In vitro translation products obtained from recombinant plasmid pTZ19/P (lanes 1 to 3), and from plasmid pGEM1/P(Bcl) by T7 transcription (lane 4) and by SP6 transcription (lane 5). The polypeptides were immunoprecipitated with anti-RS virus polyclonal serum and analysed by 15% SDS-PAGE. The arrow indicates the polypeptide translated from the internal AUG. Mr markers are shown (lane M).

Discussion

Initially mutant tsN19 was associated with a P protein defect on the basis of coordinate reversion of the ts phenotype and restoration of a P protein epitope recognized by MAb 3-5. Nucleotide sequencing of the mutant and a non-ts revertant has shown that this phenotype is the result of a Gly→Ser replacement in the predicted polypeptide sequence. Complementation group E, therefore, corresponds to the P gene of RS virus. Previously, complementation groups B and D have been assigned to the G and M genes respectively (Caravokyri & Pringle, 1991).

The paramyxovirus P and the rhabdovirus NS phosphoproteins are known to be essential components of the viral RNA polymerase complex (Emerson & Yu,
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0 5 10 20 M

Fig. 6. Internal initiation of translation in the A2 P mRNA in vivo. Replicate monolayers of BS-C-1 cells, infected with RS virus strain A2 incubated at 33 °C for 48 h, then pulse-labelled with [35S]methionine for 5 min. One culture was lysed and immunoprecipitated with anti-P MAb 8268 at the end of the pulse (lane 0). The remaining cultures were similarly treated after the chase periods indicated (min). P0 is the unphosphorylated form of the P protein (Cash, 1978), Pbrk is a breakdown product of the P protein, and Pint is the internal initiation product. Lane M contains protein M, markers.

1975; Hamaguchi et al., 1983; De & Banerjee, 1985; Deshpande & Portner, 1985). Consequently, ts lesions in these proteins have profound effects on viral mRNA synthesis at the restrictive temperature (Evans et al., 1979; Peeples et al., 1982). Early growth restriction of tsN19, as indicated by the absence of detectable protein synthesis at 39 °C, may reflect absence of synthesis of mRNA species.

Since the P protein of tsN19 presynthesized at 33 °C was stable at 39 °C, the negative phenotype at the restrictive temperature may be mediated by an effect on the functional configuration of the P protein. Direct reversion to the wild-type residue in revertant R3/6 suggests that the presence of glycine at position 172 may be essential for the structural and/or functional integrity of the P protein.

The Gly → Ser change which abolishes reactivity with MAb 3-5 is located within the Pint region, but the Pint product was not immunoprecipitated by this MAb. In conjunction with the positive reaction of MAb 3-5 with the N-terminally truncated A2 P protein (A5'-A2) this observation indicates that residues and/or correct folding in both the middle and C-terminal thirds of the protein are required for MAb binding. Therefore the 3-5 epitope may be discontinuous. Although MAb 3-5 has the ability to react with the P protein on Western blots, a property often associated with recognition of denaturation-resistant (i.e conformation-independent) antigenic determinants (reviewed by Lenstra et al., 1990), the P protein of non-segmented negative-strand RNA viruses is thought to bind poorly to SDS due to electrostatic repulsion by its acidic and phosphate groups (Gallione et al., 1981; Marnell & Summers, 1984). It is possible that binding of MAb 3-5 to P protein on Western blots is due to the inability of SDS to disrupt the conformation of the P protein effectively and/or the ability of partially denatured P protein to refold after boiling in SDS and transfer to a nitrocellulose membrane in the absence of detergent (Dunn, 1986).

Internal initiation of in vitro translation has been detected frequently in the rabbit reticulocyte lysate system, with frequencies depending on reaction conditions and RNA integrity (Gupta & Kingsbury, 1985; Dasso & Jackson, 1989; Kozak, 1989a, b). The internal initiation product (Pint) identified here was translated more efficiently in the absence of an upstream AUG or in the presence of a preceding AUG in an unfavourable context for initiation, and less efficiently in the presence of the authentic P mRNA start codon which lies in the optimal context for initiation according to the rules of the leaky scanning model (Kozak, 1986a, b).

As both the in vitro produced P transcripts and P mRNA molecules isolated from infected cells contain the same optimal P start codon, the observed difference in efficiency of internal initiation between the two kinds of transcripts (Fig. 3) could be a consequence of their different structures. Although reticulocyte lysate ribosomes exhibit relatively cap-independent binding to 5' RNA termini (Lodish & Rose, 1977; Kozak, 1989a, b), a property reflected here by the ability to translate the in vitro synthesized uncapped P RNA transcripts, the simultaneous presence of the 5' cap and 5' proximal AUG codons in mRNA molecules produced in vivo would probably have increased the efficiency of initiation at this first AUG triplet (Kozak, 1980; Dasso & Jackson, 1989). It is also possible that the absence of the 3' non-coding region from the in vitro transcripts affects secondary RNA structure, a factor contributing to the
selection of translational initiation sites (Shioda et al., 1986; Kozak, 1986a, 1989a, c; Alkhatib et al., 1988). Although any or all of the above factors may contribute to an enhanced frequency of in vitro internal initiation, the immunoprecipitation of the P polypeptide from infected cells indicates that internal initiation from the 3'-proximal AUG 148 does take place in vitro (with approximately the same efficiency as observed with in vitro translated viral mRNA; Fig. 4a, lane 2). In both Sendai virus and vesicular stomatitis virus internal initiation at a 3'-proximal AUG codon in the phosphoprotein mRNA leads to synthesis of the X and 7K proteins, respectively (Curran & Kolakofsky, 1987, 1988a, b; Vidal et al., 1988; Herman, 1986, 1987). Both polypeptides also represent the C-terminal regions of the respective phosphoproteins and have been detected in small amounts in vivo, although their functional roles remain unknown. The RS virus Pint product, consisting mainly of the acidic domains of the P protein, may play a role in infection by interacting with the basic N and/or 22K proteins.

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