PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 172, 202–206 (1983)

Identification of the Virus-Specific Proteins of Respiratory Syncytial Virus Temperature-Sensitive Mutants by Immunoprecipitation (41546)

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Abstract. The proteins of Long strain RSV and three temperature-sensitive (ts) mutants of the A2 strain were compared by pulse labeling virus-infected cells with [^{35}S]methionine and [^{3}H]glucosamine followed by analysis of the cell lysates by polyacrylamide gel electrophoresis. At the permissive temperature (30°) proteins ranging in molecular weight from 24,000 to 50,000 (VP24, VP27, VP33, VP44) could be identified. Immunoprecipitation of viral lysates by immune rabbit serum demonstrated antigenic similarity with VP27, VP44, VP50, and VP67 in all ts mutants and Long strain RSV. [^{3}H]Glucosamine labeling demonstrated glycoproteins of 90,000 (GP90) and 50,000 (GP50) in Long strain and GP90 in the ts mutants.

Respiratory syncytial virus (RSV) is the major cause of serious respiratory disease in infants. An RNA virus, RSV is a 120- to 300-nm-diameter particle (1) with spikes protruding from its envelope, one of which is felt to be a fusion protein accounting for the characteristic syncytial cytopathic effect. Initial attempts at vaccination using a formalin-inactivated RSV vaccine were unsuccessful, with vaccine recipients developing a more severe natural RSV illness than controls (2). It has been speculated that altered immunological reactivity to the wild viral infection, perhaps caused by formalin denaturation of the fusion protein in the vaccine, may have contributed to these adverse effects (3). Failure of inactivated viral vaccines led to the development and testing of cold-adapted temperature-sensitive (ts) mutants of RSV. Using 5-fluorouridine as a mutagen, Gharpure produced multiple temperature-sensitive (ts) mutants of the A2 (Australian) strain of RSV (4). These ts mutants have been well characterized with regard to their growth characteristics and genetic stability at the permissive (30°) and restrictive (37°) temperatures, and have been categorized into at least seven complimentation groups (A through G) (5–8). To date the ts-1 and ts-2 mutants have been evaluated as possible vaccine strains (9, 10).

In order to be an effective vaccine, an attenuated ts mutant should replicate in the vaccine recipient and present an antigenic challenge similar to the wild-type virus. This would require ts mutant synthesis of structural proteins which are antigenically similar to the parent strain. We therefore compared the presence of the proteins of ts-1, ts-2, and ts-7 grown in tissue culture at the permissive and restrictive temperature to the Long strain of RSV grown at 30° and 37°, respectively. In addition, those proteins capable of immunoprecipitation with rabbit hyperimmune serum were compared using radiolabeling of viral proteins and analysis of polyacrylamide gel electrophoresis (PAGE), a technique we have previously utilized to demonstrate Long strain viral proteins (11).

Materials and Methods. *Cells and virus.* Hep-2 cell lines were grown in Eagle's minimum essential medium (MEM) containing 5% fetal calf serum (FCS). Stock RSV Long strain and ts-1, ts-2, and ts-7 (all obtained from R. Chanock) were grown at 30° (except RSV, grown at 37°) in Hep-2 cells to titers of 2–6 $\times 10^5$ plaque-forming units (PFU)/ml, and frozen at −70° until use.

Antibody to RSV. Antibody to RSV was produced in New Zealand white rabbits by injection of purified Long strain virus grown in bovine embryonic kidney cells as previously described in detail (11). This antibody has no reactivity against Hep-2 cells.

Radiolabeling of RSV. Hep-2 cells, grown in six-well Costar plates, were infected with each strain at the following multiplicities of infection (m.o.i.): Long strain RSV (m.o.i.

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0037-9727/83/020202-05$01.50/0

= 1), ts-1 (m.o.i. = 0.2), ts-2 (m.o.i. = 0.2), and ts-7 (m.o.i. = 0.1). After a 90-min absorption period at room temperature the medium was aspirated and replaced with 3 ml of fresh MEM containing 5% agammaglobulin fetal calf serum, and incubated in 5% CO_2 at 30° or 37°. When early CPE was detected, the medium was aspirated and replaced with methionine-free MEM, without FCS, containing [^{35}S]methionine at a concentration of 10 μCi/ml (Amersham Corp.). Between 3 and 24 hr later the medium was aspirated. The cell sheet was lysed with 0.5 ml RIPA buffer (0.01 *M* Tris–HCl, pH 7.4, 0.15 *M* NaCl, 1% sodium desoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate) containing 1 m*M* phenylmethylsulfonyl fluoride (PMSF). This lysate was then analyzed by PAGE.

Similar labeling techniques were used to radiolabel with [^{3}H]glucosamine, except that the labeling medium was MEM with 50% of the usual dextrose concentration supplemented with [^{3}H]glucosamine, 10–50 μCi/ml (Amersham Corp.).

Immunoprecipitation. Immunoprecipitation and polyacrylamide gel electrophoresis were carried out by previously described methods (11).

Results. *Identification of viral proteins at the permissive temperature (30°).* Growth of Long strain RSV and the temperature-sensitive mutants ts-1, ts-2, and ts-7 in HEp-2 cells at 30° resulted in the synthesis of four presumed viral proteins which were not seen in uninfected cells (see Figs. 1 and 2). Three of these proteins had molecular weight (MW) of 44,000, 33,000, and 27,000 daltons and were seen with all RSV strains. The fourth protein of 24,000-dalton molecular weight was seen

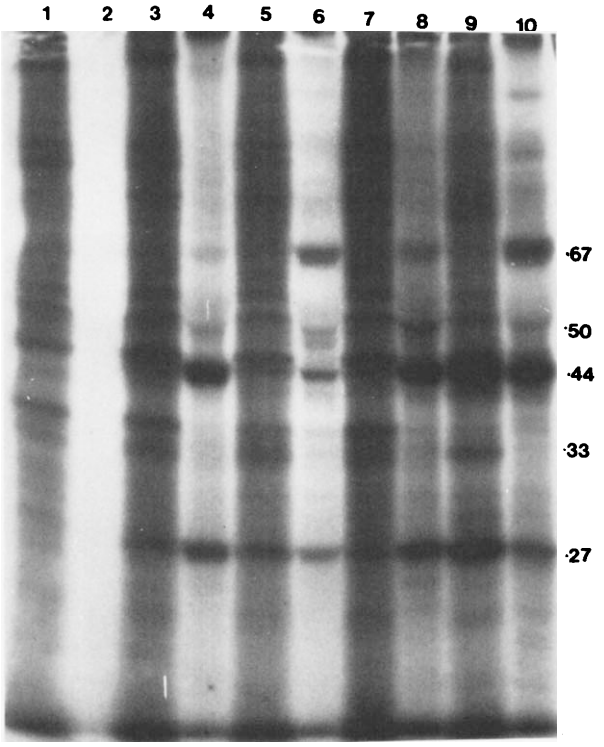

FIG. 1. Proteins of Long strain RSV and the ts mutants grown at the permissive temperature (30°). Virus was grown at 30° for 48 hr, then pulse labeled with [^{35}S]methionine, lysed in RIPA buffer, immunoprecipitated with rabbit anti-RSV sera, electrophoresed on a 12% polyacrylamide gel, and fluorographed. Samples applied to the gel were as follows: HEp-2 cell lysate (1), immunoprecipitate of HEp-2 (2), Long strain RSV lysate (3), immunoprecipitate of Long strain (4), ts-1 lysate (5), immunoprecipitate of ts-1 (6), ts-2 lysate (7), immunoprecipitate of ts-2 (8), ts-7 lysate (9), and immunoprecipitate of ts-7 (10).

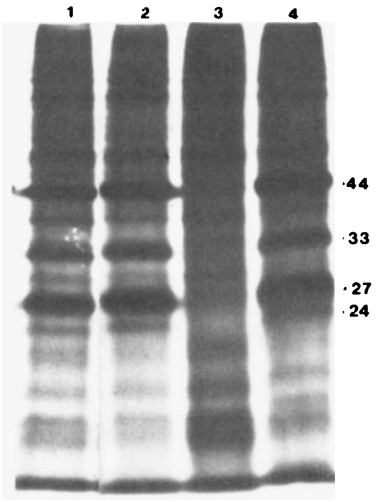

FIG. 2. Proteins of Long strain RSV and the ts mutants grown at the permissive temperature (30°), as in Fig. 1, but run on a 10% polyacrylamide gel. Samples applied to the gel are ts-1 lysates (1), ts-7 lysates (2), HEp-2 lysates (3), and Long strain RSV lysates (4). This gel resolves a viral peptide with MW 24,000 daltons.

only in the Long strain, ts-1, and ts-7; this latter polypeptide is best resolved on a 10% polyacrylamide gel as shown in Fig. 2.

Immunoprecipitation of the cell culture lysates with specific anti-RSV rabbit immune serum revealed viral-specific polypeptides of MW 67,000, 50,000, 44,000, and 27,000 daltons for each of the ts mutants and Long strain (Fig. 1).

Identification of viral-specific proteins at the restrictive temperature (37°). At the restrictive temperature ts-2 was the only temperature-sensitive strain which readily labeled with [^{35}S]methionine, demonstrating polypeptides of 50,000, 44,000, 33,000, and 27,000 daltons (figure not shown). The proteins of ts-1 and ts-7 were poorly discerned at 37°. In an attempt to radiolabel ts-1 and ts-7 at 37°, shift-up experiments were done. In these experiments the virus was initially incubated at 30° for 48 hr. Cultures were then incubated at 37° and [^{35}S]methionine was added at various time points after shift-up to 37°. However, even after shift-up, only trace amounts of proteins of MW 50,000, 44,000, and 27,000 daltons could be identified for ts-1 and ts-7 (Fig. 3). Long strain and ts-2 proteins, by contrast, were well labeled during shift-up experiments demonstrating polypeptides with MW of 44,000, 33,000, 27,000 and 24,000 daltons. Additionally with both Long strain and ts-2 a protein of MW 10,000 daltons was also noted in these experiments (Fig. 3).

Identification of viral glycoproteins at permissive temperature. Previous work had shown that Long strain RSV contained two virally specified glycopeptides of 50,000- and 90,000-dalton molecular weight; however, the 90,000-dalton polypeptide was labeled only with [^{3}H]glucosamine but not with [^{35}S]-methionine (11). After [^{3}H]glucosamine labeling of Long strain RSV, ts-1, ts-2, and ts-7 at 30°, a 90,000-dalton polypeptide was seen with all strains (Fig. 4). However, at 37° only in the Long strain RSV cultures was the

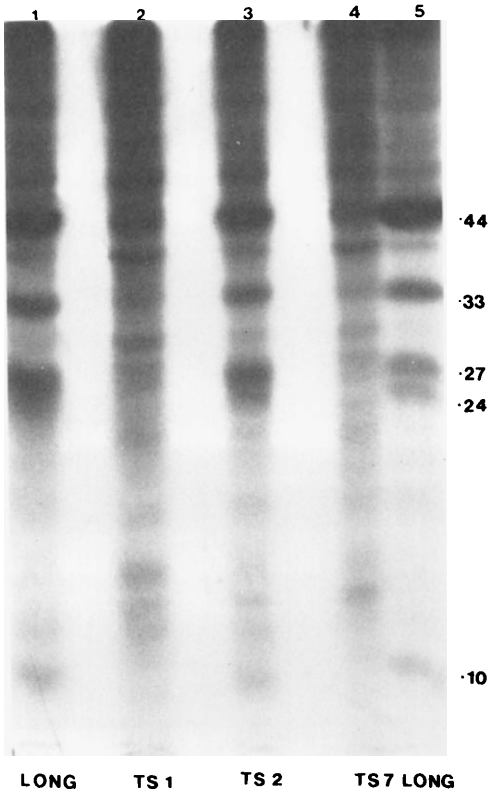

FIG. 3. Protein of Long strain and ts mutants after step-up to the restrictive temperature (37°). In this experiment virus was incubated at 30° for 48 hr, then stepped up to 37° and pulse labeled after 3 hr at 37°. Long strain (lanes 1 and 5) and ts2 (lane 3) demonstrate proteins MW 44K, 33K, 27K, 24K, and 10K. ts1 (lane 2) and ts7 (lane 4) are poorly labeled.

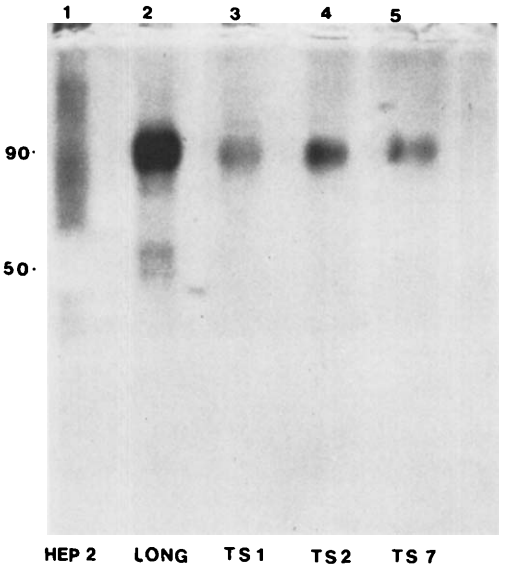

FIG. 4. [^{3}H]Glucosamine-labeled protein of Long strain RSV and the ts mutants grown at the permissive temperature (30°). Virus was grown at 30° in HEp-2 cells for 48 hr, then labeled with [^{3}H]glucosamine, lysed with RIPA buffer, electrophoresed on a polyacrylamide slab gel and fluorographed. Samples applied to gel are cell lysates of HEp-2 (1), Long strain RSV (2), ts-1 (3), ts-2 (4), and ts-7 (5).

90,000-dalton glycopeptide seen (figure not shown). In addition, at 30° a 50,000-dalton glycoprotein was seen in the Long strain RSV but not with the ts mutants.

Discussion. These labeling experiments were carried out with three ts mutants and the Long strain of RSV. The ts mutants, ts-1, ts-2, and ts-7 have previously been categorized into complimentation groups A, B, and C, respectively. Prior to the labeling experiments plate complimentation studies were carried out to confirm the separate complimentation groups of each ts mutant. In addition the inability of ts-2 to form syncytia at both 30° and 37° was documented.

The labeling studies presented here demonstrate that at the permissive temperature (30°), the temperature-sensitive mutants and Long strain produce proteins of similar size with the exception of the 24,000-dalton protein for ts-2. However, in the step-up labeling experiment this protein was seen for ts-2. The significance of this is not clear. Immunoprecipitation of these proteins with hyperimmune rabbit sera demonstrated similar proteins consistently. Using [^{3}H] glucosamine as the label, a 90K MW glycoprotein, presumably a surface viral glycoprotein, was noted in all ts mutants and Long strain at the permissive temperature, whereas the 50K MW glycoprotein could only be demonstrated for Long strain. However, using [^{35}S]methionine as the label, all ts mutants demonstrated a 50K MW protein, suggesting that the absence of labeling by [^{3}H]glucosamine may have been a technical problem related to the relatively lighter labeling of ts mutants with [^{3}H]-glucosamine, as seen in Fig. 4.

These data would suggest that at the permissive temperature of 30°, which approximates human nasal temperature, these ts mutants produce analogous proteins with similar antigenic reactivity as the Long strain of RSV as measured by their ability to be immunoprecipitated by anti-RSV antibody. This has potentially important implications in the use of ts mutants as vaccine strains, especially in view of the adverse results with the inactivated RSV vaccine. It has been speculated that the more severe natural RSV infection seen following vaccination with formalin-inactivated virus may have been due to the inactivation of the antigenicity of the fusion protein by formalin. This could result in the failure of vaccines to produce antibodies to the fusion protein, and thus alter their immunological interaction with natural viral infection. This would be most relevant to the nonsyncytial forming mutant, ts-2, which presumably produces a nonfunctional fusion protein. Thus far, it has not been proven which of RS viral proteins is the fusion protein.

In addition these studies give additional support to the identifications of the proteins of RSV. Labeling with [^{35}S]methionine of the ts mutants demonstrated proteins of 67,000, 50,000, 44,000, 33,000, 27,000, 24,000, and 10,000 daltons, while labeling with [^{3}H]glucosamine demonstrates a glycoprotein of 90,000 and 50,000 daltons.

Recently, Pringle has demonstrated similar protein synthesis by these ts mutants; however, the immunologic reactivity with anti-RSV antibody was not documented by specific immunoprecipitation (12). Several differences between their studies and ours are evident. They did not demonstrate a protein

of MW 67,000 daltons; we could only demonstrate this protein by specific immunoprecipitation, a technique they did not use. In addition we did not identify a protein of MW 200,000. This may be due to the poor resolving ability of 10–12% polyacrylamide gels at that molecular weight.

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Received July 19, 1982. P.S.E.B.M. 1983, Vol. 172.