

Immunoprecipitation as a Tool for Studying Humoral Immunity of Natural and Experimental Hosts of *Herpesvirus saimiri*¹

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ABSTRACT

Herpesvirus saimiri strain 11 and attenuated *H. saimiri* strain 11 proteins synthesized during the lytic cycle of virus replication were used for immunoprecipitation with various sera from natural (*Saimiri sciureus*) and experimental (*Saguinus nigricollis*, *Saguinus fuscicollis*, *Aotus trivirgatus*, New Zealand White rabbits) hosts. The analysis of the precipitates separated in sodium dodecyl sulfate:polyacrylamide gels revealed that in tumor-developing animals a specific set of viral polypeptides were precipitated, which were not precipitated by sera obtained from the natural host *Saimiri sciureus*. Using labeled proteins from *H. saimiri* 11 and its attenuated strain, respectively, a difference was shown after precipitation with a serum raised against infected cell proteins of *H. saimiri* 11.

INTRODUCTION

Marmosets of the genus *Saguinus* (*Saguinus oedipus*, *Saguinus nigricollis*, *Saguinus fuscicollis*) are highly susceptible to tumor induction by *Herpesvirus saimiri* and develop a fatal, rapidly growing neoplastic disease (3, 11) following virus infection. Owl monkeys (*Aotus trivirgatus*) show a similar course of disease. The appearance of tumors in these animals is often delayed, and about 20% of the owl monkeys infected with *H. saimiri* do not develop tumors (7, 10). *H. saimiri*-infected NZWR³ show a disease pattern similar to that of primates; the incidence of neoplastic disease after infection with *H. saimiri* varies between about 20 and 75% in different studies (1, 2, 16). In squirrel monkeys (*Saimiri sciureus*), the natural hosts of *H. saimiri* are apparently infected and are lifelong virus carriers.

In our study, we tested the specificity of antibodies in the various experimental and natural hosts by immunoprecipitation of viral polypeptides obtained from OMK cells infected with *H. saimiri* strain 11 or an attenuated mutant of *H. saimiri* 11 (*H. saimiri* 11 att) (17).

MATERIALS AND METHODS

Cells. OMK cells were cultured in 32-ounce glass prescription bottles or plastic Petri plates using MEM (Earle's salts; Grand Island Biological Co.) supplemented with 20 mM glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% heat-inactivated fetal calf serum (Seromed, Munich, Germany).

Virus. *H. saimiri* 11 was originally obtained from B. Fleckenstein

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³ The abbreviations used are: NZWR, New Zealand White rabbit; OMK cells, owl monkey kidney cells; MEM, minimal essential medium; PFU, plaque-forming units; i.c., intracutaneously; SDS, sodium dodecyl sulfate; p152, protein with a molecular weight of 152,000 (other proteins are designated similarly); p.i., postinfection; EA, early antigen.

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(University of Erlangen, Erlangen, Germany), the attenuated *Herpesvirus saimiri* (*H. saimiri* 11 att) mutant was a gift of F. Deinhardt (Max von Pettenkofer-Institute). Cells were infected with *H. saimiri* at 1 to 2 PFU/cell; after an adsorption period of 2 hr at room temperature, the cultures were replenished with MEM supplemented with 2% heat-inactivated fetal calf serum and phorbol-12-myristate-13-acetate (20 µg/ml; Sigma, Munich, Germany) (12) and incubated at 34° until a pronounced cytopathic effect became visible (12).

Sera. The sera of squirrel monkeys (Ss 1, Ss 2, Ss 3) and owl monkeys (At 1, At 2, At 3) were a gift of L. Falk, New England Primate Research Center, Southborough, Mass. The *S. nigricollis* serum (Sn 1) was obtained from a monkey which had died of a neoplastic disease 7 weeks after inoculation i.m. with 1 × 10⁶ PFU *H. saimiri* 11. The *S. fuscicollis* serum (Sf 1) was infected with *H. saimiri* 11 att and developed a lifelong virus carrier status without showing any signs of disease; the serum was obtained 7 years after inoculation (gift of F. Deinhardt). The rabbit (NZWR1) was inoculated i.v. with 1 × 10⁶ PFU *H. saimiri* 11 and died 3 weeks after inoculation. A serum against viral structural proteins was obtained from a rabbit (R 1) inoculated i.c. with multiple doses of purified *H. saimiri* 11 particles in the presence of complete Freund's adjuvant. A serum against virus-induced infected cell proteins was obtained from a goat inoculated with multiple doses of *H. saimiri* 11-infected cells (gift of F. Deinhardt).

Labeling of Proteins Synthesized by Infected OMK Cells and Preparation of Cell Extracts. OMK cells were infected at 90% confluence with *H. saimiri* 11 or *H. saimiri* 11 att and labeled at various times after infection with [³⁵S]methionine (20 µCi/ml; Amersham Buchler, Braunschweig, Germany) in methionine-free MEM. A set of early proteins was accumulated by the addition of azetidine (500 µg/ml; Sigma). At the end of the labeling period, cells were rinsed 3 times with cold phosphate-buffered saline (140 mM NaCl, 3 mM KCl, 0.75 mM Na₂PO₄, 1 mM CaCl₂, 5 mM MgCl₂) to stop incorporation of amino acids, solubilized in immunoprecipitation buffer [0.5 ml/1 × 10⁶ infected cells, 1% Triton X-100, 0.1% SDS, 0.137 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10% glycerol, 20 mM Tris-HCl (pH 9.0), 0.01% NaN₃, phenylmethylsulfonyl fluoride (1 µg/ml; Sigma)], and disrupted by sonication (Branson sonifier). The extracts were clarified by centrifugation at 100,000 × g for 30 min at 4° and stored at -20°.

Immunoprecipitation and Electrophoresis. In order to remove antibodies against cellular proteins, 10 µl of each serum were incubated with 0.5 ml of an extract of uninfected, unlabelled OMK cells (1 × 10⁷ cells/ml, prepared as described above) overnight at 4°. Protein A-Sepharose beads (3 mg; Pharmacia, Freiburg, Germany), preswollen in 100 µl immunoprecipitation buffer, were added, and the reaction was incubated at 4° for another 2 hr. Labeled protein extract (0.3 ml) was added, and the immunocomplexes were allowed to form during a 3-hr incubation period at room temperature. The beads were washed with immunoprecipitation buffer until supernatants contained no detectable radioactivity. The washed beads were resuspended in electrophoresis sample buffer [50 mM Tris-HCl (pH 7.0), 2% SDS, 5% mercaptoethanol, 3% sucrose:bromophenol blue] and heated to 100° for 5 min to dissociate the immune complexes. The supernatant was analyzed on 10% SDS:polyacrylamide gels; 10,000 cpm were applied per slot, when available. Electrophoresis was done in a discontinuous buffer system (9) on slab gels. The separating gel contained 10% acrylamide, 0.26% dialyltartardiamide, 0.1% SDS, and 375 mM Tris-HCl (pH 8.5). Polymerization was initiated by adding ammonium persulfate to a final concentra-

tion of 0.14 mm and *N,N,N',N'*-tetraethylmethylenediamine to a final concentration of 0.035%. The stacking gel contained 3% acrylamide, 0.08% diallyltartardiimide, 0.1% SDS, and 120 mM Tris-HCl, pH 7.0. The upper and lower buffer tanks contained 25 mM Tris-HCl (pH 8.5), 192 mM glycine, and 0.1% SDS. Electrophoresis was performed at a constant current of 5.3 mA/sq cm until the leading front reached the end of the gel. Gels were fixed and stained in water:acetic acid:isopropyl alcohol (4:1:1), destained in water:acetic acid:isopropyl alcohol (8:1:1), and dried onto Whatman No. 2MM filter paper under vacuum. All chemicals were purchased from Bio-Rad (Munich, Germany); high-molecular-weight standards from the same company were used as markers. Gels were exposed to ³H-Ultronfilm (LKB, Gräfelfing, Germany).

RESULTS

Precipitation with *Saimiri sciureus* Sera. *H. saimiri*-infected cell proteins were labeled at early (6 to 8 hr p.i.), middle (15 to 17 hr p.i.), and late (24 to 26 hr p.i.) times and in the presence of azetidine (500 µg/ml) with [³⁵S]methionine. The labeled polypeptides were precipitated with 3 different sera of squirrel monkeys (Ss 1, Ss 2, Ss 3). In azetidine-treated cells (Fig. 1) and at early times after infection, only a few viral proteins could be precipitated: p152, p53, p50, and p28 (Fig. 1) and p152, p97, p53, and p46 (Fig. 2). When proteins were labeled at middle (not shown) and late times (Fig. 3) after infection, a subset of viral polypeptides was identified, composed of mostly viral structural proteins (13) produced at the end of the lytic cycle: p195, p152, p146, p135, p127, p123, p106, p97, p88, p67, p61, p53, p50, p28 (12). A serum against virion proteins produced in rabbits (R 1) was used as positive control. All sera were tested with mock-infected cells to rule out unspecific binding (data not shown).

Precipitation with Sera of Experimental Hosts. [³⁵S]Methionine-labeled proteins were precipitated with 3 different owl monkey sera (At 1, At 2, At 3), with one *S. nigricollis* (Sn 1) serum, and one NZWR (NZWR 1) serum from animals which were dying of neoplastic disease after infection with *H. saimiri* 11. At middle and late times (Fig. 3) after infection, sera from the experimental hosts precipitated a limited number of viral proteins: p152, p127, p115, p80, p55–57 (p55 for *A. trivirgatus*, p57 for *S. nigricollis* and NZWR), p53, p50, p28. p146 and p135 were precipitated with some owl monkey sera only in very reduced amounts; p195, p123, p106, p97, p88, p67, and p61 were never seen. Three proteins, however, were not found to be precipitated with the sera of the natural hosts: p115, p80, p55–57. p115 and p55–57 are already synthesized at an early stage after infection [6 to 8 hr p.i. (Fig. 2)]. In the presence of azetidine, p115 is not synthesized (Fig. 1).

Precipitation with Serum of an Animal Infected with *H. saimiri* 11 att. *H. saimiri* 11-infected cell proteins were precipitated with a serum of a *S. fuscicollis* (Sf 1), which was persistently infected with *H. saimiri* 11 att. The protein profiles obtained after precipitation were very similar to those obtained with the sera of *Saimiri sciureus*, most of the proteins being structural polypeptides (Figs. 1 to 3).

Comparison of *H. saimiri* 11- and *H. saimiri* 11 att-infected Cell Proteins. All sera from the natural and experimental hosts showed no difference in the protein profiles of *H. saimiri* 11- and *H. saimiri* 11 att-infected cell proteins. By immunoprecipitation with a serum prepared against *H. saimiri* 11-infected cell proteins in a goat (Fig. 4), one polypeptide was precipitated in *H. saimiri*

11-infected cells which was not found in *H. saimiri* 11 att-infected cells; the protein had a molecular weight of about 55,000.

DISCUSSION

In this study, we determined the specificity of antibodies in various experimental (*S. nigricollis*, *S. fuscicollis*, *A. trivirgatus*, NZWR) and natural (*Saimiri sciureus*) hosts by immunoprecipitation of proteins. These polypeptides were obtained from OMK cells infected with *H. saimiri* 11 or *H. saimiri* 11 att in the presence of [³⁵S]methionine at different times after infection. In the natural hosts, most antibodies are directed against late viral polypeptides (Fig. 3), which are mostly structural proteins of the *H. saimiri* virion (13): p195, p152, p146, p135, p127, p123, p106, p97, p88, p67, p61, p53, p50, p28.

A very similar picture was obtained with a serum from a *S. fuscicollis* (Sf 1), which was infected with *H. saimiri* 11 att and developed a lifelong virus carrier status. *H. saimiri* 11 att (17) is reported to be nononcogenic in different marmoset species, induces a latent or persistent infection, and protects animals against challenge inoculation with *H. saimiri* 11 (4, 18, 19). Thus, the etiological behavior of *H. saimiri* 11 att in marmosets is very similar to that of *H. saimiri* 11 in *Saimiri sciureus* monkeys. This fact could be demonstrated by the results obtained for the immunoprecipitation experiments. The sera of squirrel monkeys infected with the oncogenic wild type showed the same antibody pattern as that obtained from a marmoset infected with *H. saimiri* 11 att.

Sera from experimental hosts infected with *H. saimiri* 11 which had died from a neoplastic disease showed after immunoprecipitation that their antibody specificity was different from that obtained from the natural host but very similar among the different species (NZWR, owl monkey, marmoset). With the sera of all these hosts, 3 proteins were precipitated which were never precipitated with sera from squirrel monkeys: p115, p80, and p55 in owl monkeys; and p57 in *S. nigricollis* and NZWR, respectively. The slight difference in the molecular weight (p55–p57) may be due to a different modification of the protein in the various hosts with the antibodies directed against the modification. p115 and p55–p57 were synthesized at an early stage after infection (Fig. 2); the synthesis of p115, however, was inhibited by the treatment with azetidine (Fig. 1) and thus may belong to a second group of early proteins.

Since all animals developing a *H. saimiri*-induced neoplastic disease are reported to have high anti-EA titers (8, 14, 15) which increase with the development of malignant tumors, one might conclude that the 3 proteins p115, p80, and p55–57 are components of the EA complex. In the natural hosts, the anti-EA titers were found to decline ten months after the primary infection; this is very similar to the serological data for Epstein-Barr virus in humans, where rising anti-EA titers are a prognostically unfavorable sign indicating neoplastic proliferation (5, 6).

Between the protein profiles obtained from *H. saimiri* 11- and *H. saimiri* 11 att-infected cell proteins precipitated with squirrel monkey, owl monkey, marmoset, and NZWR sera, respectively, no differences could be detected. Precipitation with a goat serum against *H. saimiri* 11-infected cells produced, however, one protein that could be identified in *H. saimiri* 11-infected cells which was not synthesized in *H. saimiri* 11 att-infected cells (Fig. 4). The molecular weight of the protein was found to be 55,000.

This protein may either be a viral protein, which is deleted in *H. saimiri* 11 att, or a cellular polypeptide the synthesis of which is not induced during infection with *H. saimiri* 11 att. As a third possibility, the differences in the protein patterns may be due to a different protein modification of *H. saimiri* 11- and *H. saimiri* 11 att-induced polypeptides.

The characterization of the polypeptides which present themselves in the various experimental and natural hosts as antigens may be a step towards a better understanding of the regulatory mechanisms involved in the development of the *H. saimiri*-induced malignant disease. At least these experiments show that tests for antibodies directed against specific antigens could be used to discriminate between tumor-bearing and non-tumor-bearing hosts and that antibodies reacting with other antigens are of use for a nondiscriminating diagnosis of the immune status. Although this may be of greatest importance for hosts infected with *H. saimiri*, similar work may prove very useful for the diagnosis of infectious or neoplastic disease caused by Epstein-Barr virus and other herpesviruses in humans.

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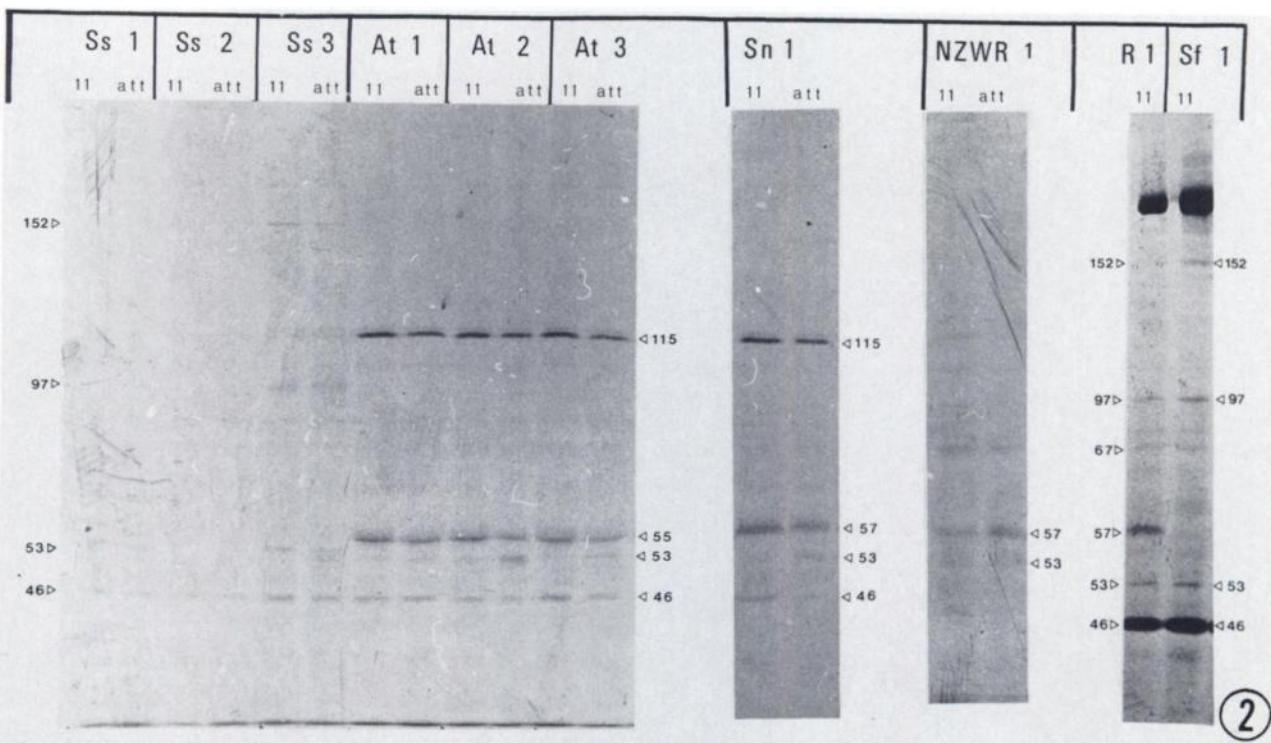
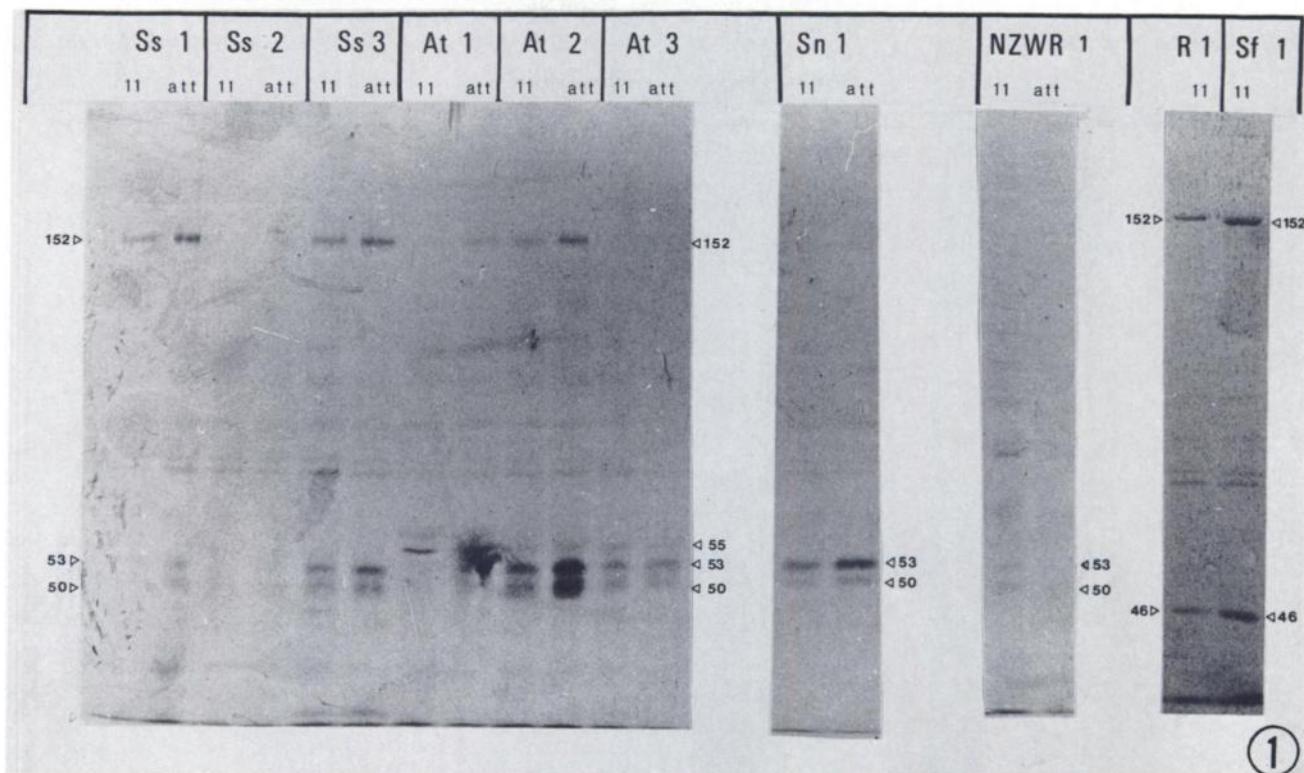


Fig. 1. *H. saimiri*-infected OMK cells treated with azetidine (500 µg/ml), labeled with [³⁵S]methionine (20 µCi/ml) from 24 to 26 hr after infection, and immunoprecipitated with the sera indicated. 11, *H. saimiri*-11 infected cells; att, *H. saimiri* 11 infected cells; Ss 1, Ss 2, Ss 3, sera of *Saimiri sciureus*, infected with *H. saimiri* 11; At 1, At 2, At 3, sera of *A. trivirgatus*, infected with *H. saimiri* 11; Sn 1, serum of *S. nigricollis*, infected with *H. saimiri* 11; Sf 1, serum of *S. fuscicollis*, infected with *H. saimiri* 11 att; NZWR 1, serum of a NZWR, infected with *H. saimiri* 11; R1, rabbit serum against structural proteins of *H. saimiri* 11.

Fig. 2. *H. saimiri*-infected OMK cells, labeled with [³⁵S]methionine (20 µCi/ml) from 6 to 8 hr after infection, and immunoprecipitated with the sera indicated. For identification of abbreviations, see legend to Fig. 1.

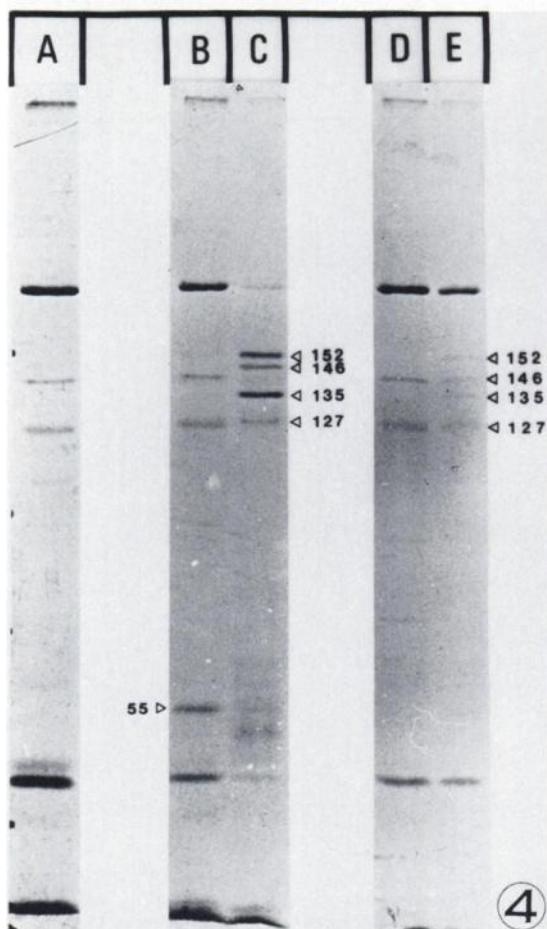
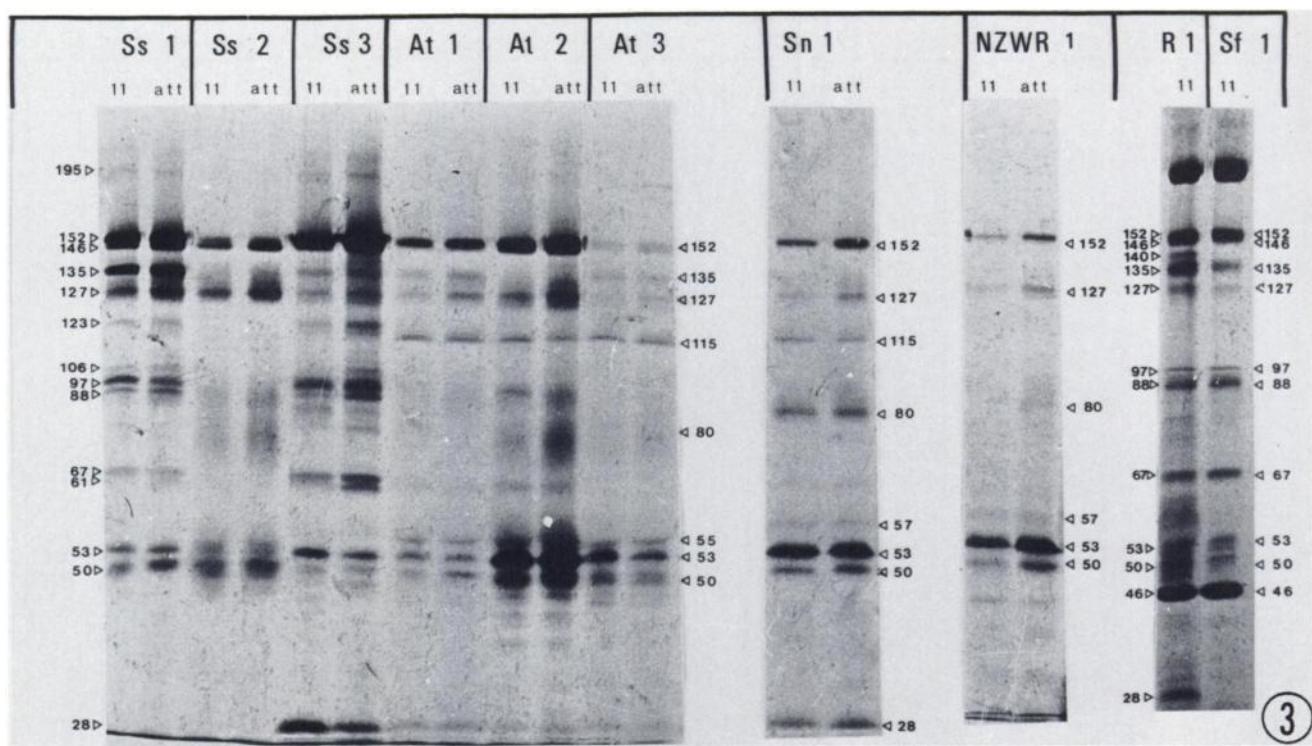


Fig. 3. *H. saimiri*-infected OMK cells, labeled with [³⁵S]methionine (20 μ Ci/ml) from 24 to 26 hr after infection, and immunoprecipitated with the sera indicated. For identification of abbreviations, see legend to Fig. 1.

Fig. 4. OMK cells infected with *H. saimiri*, labeled at various times after infection with [³⁵S]methionine (20 μ Ci/ml), and immunoprecipitated with a goat serum directed against *H. saimiri* 11-infected cell proteins. A, mock-infected cells, labeled 24 to 26 hr after mock infection; B, *H. saimiri* 11-infected cells, labeled 8 to 10 hr after infection; C, *H. saimiri* 11-infected cells, labeled 24 to 26 hr after infection; D, *H. saimiri* 11 att-infected cells, labeled 8 to 10 hr after infection; E, *H. saimiri* 11 att-infected cells, labeled 24 to 26 hr after infection.