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Preferential virosomal location of underphosphorylated H5R protein synthesized in vaccinia virus-infected cells

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The phosphorylation state of vaccinia virus (VV) protein H5R synthesized in infected cells was investigated by two-dimensional gel electrophoresis. Most of the H5R protein was underphosphorylated (pI 5·9 to 6·8) and, on centrifugation of cell lysates, was associated with virosomes sedimenting with nuclei. However, about a quarter of the H5R protein synthesized was highly phosphorylated (pI 5·5), and this was the major form of the H5R protein present in cytoplasmic extracts. Immunofluorescence of VV-infected cells in the absence of DNA replication showed that underphosphorylated H5R protein, specifically recognized by antibody, was abundantly distributed throughout the cytoplasm but also present in punctate particles, whereas most of the B1R protein detected was in the punctate particles. Late gene expression was not required for the H5R protein to accumulate in virosomes – viral DNA synthesis was sufficient. The different phosphorylation states and cytological locations of the H5R protein suggest it has multiple roles in VV development.

Introduction

Vaccinia virus (VV), a member of the *Poxviridae*, replicates its 190 kb DNA genome and expresses its genes exclusively in the cytoplasm of infected cells (Moss, 1996). It has long been known that VV DNA replication and virus assembly occur in granular cytoplasmic structures, designated virosomes or virus factories (Cairns, 1960). Virosomes were isolated as particulate material containing replicating VV DNA complexes (Dahl & Kates, 1970) and their protein composition was investigated in the 1970s. Several major components have been described (Sarov & Joklik, 1973; Polisky & Kates, 1972, 1975, 1976; Nowakowski *et al.*, 1978*a*, *b*) including a major early phosphoprotein of 36 kDa phosphorylated on threonine residues, which has been identified as the product of the H5R gene (Beaud *et al.*, 1995). The H5R gene was previously reported to encode the major envelope p35 antigen (Gordon *et al.*, 1988, 1991). However, the targeting of H5R protein toward viral membranes is doubtful because it was recently found, by direct microsequencing of purified p35 protein and by cloning in *E. coli*, that the immunodominant p35 membrane protein is actually encoded by gene H3R (Zinoviev *et al.*, 1994). Furthermore, Zinoviev *et al.* (1994) found that the product of the H5R gene (designated H6R in their paper) was absent from mature virions.

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Methods

■ **Metabolic labelling.** Monolayers (10 cm^2) of almost confluent BSC-40 cells were infected with WR VV at a ratio of 10 p.f.u. per cell. After an adsorption period of 1 h, the infected cells were washed with PBS and radioactive medium was added [MEM without methionine and cysteine, containing 20 mM HEPES pH 7·2, 10% dialysed newborn calf serum and 75 μCi/ml Tran^{35}S-Label (ICN)].

■ **Preparation of total cell lysates.** The cells were washed twice with PBS and then scraped from the wells with 0·4 ml extraction buffer (30 mM Tris–HCl pH 7·5, 1 mM EDTA, 1 mM EGTA, 40 mM glycerophosphate, 1 mM sodium vanadate, 40 μg/ml phenylmethylsulfonyl fluoride and 20 μg/ml each of leupeptin, aprotinin and pepstatin). Total cell lysates were prepared by ultrasonication (3 × 20 s) with a 3 mm microtip and precipitation at −20 °C with 5 vols acetone.

■ **Preparation of cytoplasmic and nuclear extracts.** Cells were removed from the monolayer as described above and then lysed in extraction buffer containing 0·5% NP40. The mixture was vortexed, incubated for 5 min at 0 °C and centrifuged for 10 min at 12500 ***g*** in a microcentrifuge in a cold room. The supernatant (cytoplasmic extract) was precipitated overnight at −20 °C with acetone. The pellet (containing the nuclei) was suspended in 0·4 ml extraction buffer, ultrasonicated and precipitated with acetone as described above. For two-dimensional gel electrophoresis, the acetone pellets were dried and dissolved in urea sample buffer [9·5 M urea, 1% NP40, 4·5% Ampholine pH 5–8 (Pharmacia), 2·5% Ampholine pH 3·5–9·5 and 50 mM dithio-

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threitol]. *In vivo* ^{32}P-labelling of the H5R protein and two-dimensional gel electrophoresis (O'Farrell, 1975) were carried out as previously described (Beaud *et al.*, 1995).

Results and Discussion

Characterization by two-dimensional gel electrophoresis of different forms of the ^{35}S-labelled H5R protein synthesized in cells infected with VV

We had previously shown by peptide sequencing that the H5R protein is a 36 kDa protein with multiple forms of different isoelectric point: approximately 5·5, 5·7, 5·9 and 6·3 (Beaud *et al.*, 1995). Hence we were able to investigate the phosphorylation state of the H5R protein by two-dimensional gel electrophoresis of ^{35}S-labelled proteins labelled *in vivo*. We first identified the H5R protein among the ^{35}S-labelled proteins separated by two-dimensional gel electrophoresis and showed that several 36 kDa ^{35}S-labelled proteins synthesized in infected cells co-migrated with previously purified H5R protein (Beaud *et al.*, 1995). As shown in Fig. 1(*a*), the different forms of ^{35}S-labelled 36 kDa protein with pIs of 5·5, 5·7, 5·9 or 6·3 co-migrated exactly with authentic non-radioactive H5R protein which, as it was added in excess, was identifiable as the material stained with Coomassie blue. In other analyses (without adding excess unlabelled H5R protein) two major spots were evident, one of pI 5·5 and the other either of pI 6·3 (as in Fig. 1) or 6·8 (as shown in Fig. 2). This slightly different pattern may be due to a limited dephosphorylation of the H5R protein occurring during preparation of the cell extracts. As expected, the multiple forms of the H5R protein with pIs of 5·5 to 6·3 corresponded to proteins phosphorylated *in vivo*, because we also observed an identical migration of ^{32}P-labelled 36 kDa protein (obtained after ssDNA cellulose chromatography) with authentic H5R protein (Fig. 1*b*).

We also used an immunological procedure to characterize the H5R protein, using a rabbit antiserum prepared against an amino-terminal peptide of the H5R protein (amino acids 2–16). Immunoblotting experiments revealed that this antibody recognized only underphosphorylated forms of the H5R protein: the most acidic form, with a pI of 5·5, did not react at all with the antibody whereas the forms with pIs of 6·3 (present in a lower amount in the purified preparation of the H5R protein; Fig. 1*c*) or 6·8 (see Fig. 2) were the most efficiently recognized (Fig. 1*c*). Because the H5R protein is phosphorylated on threonine residues *in vivo* (Nowakowski *et al.*, 1978*a*) and *in vitro* by the B1R kinase (Beaud *et al.*, 1994), the specificity of the H5R protein antibody for the underphosphorylated forms suggests that at least one of the threonine residues at positions 6, 11 or 16 is phosphorylated in the most acidic forms of the H5R protein (pI 5·5–5·7).

Phosphorylation state and cytological location of the H5R protein

Next, we analysed by two-dimensional gel electrophoresis the phosphorylation state of the ^{35}S-labelled H5R protein

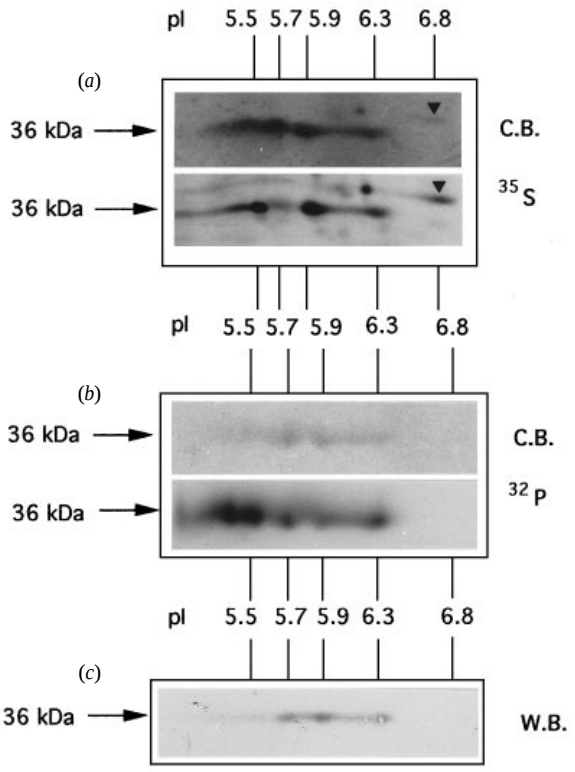

Fig. 1. Characterization of radioactive H5R protein synthesized *in vivo*. Cytoplasmic extracts of cells infected with VV and labelled with Tran^{35}S-Label (*a*) or [^{32}P]phosphate (*b*) were prepared as described in Methods. Then, 4 μg of previously purified H5R protein (Beaud *et al.*, 1995) was added to each cytoplasmic extract and the mixture was analysed by two-dimensional gel electrophoresis, as described in Methods. The relevant portion of the dried gel stained with Coomassie blue (C.B.) and the corresponding autoradiograms shown below (^{35}S, ^{32}P). (*c*) Western blot (W.B.) of 4 μg of the purified H5R protein, also separated by two-dimensional gel electrophoresis. Only the relevant parts of gels are shown. The arrowheads in (*a*) indicate an abundant cellular protein, presumably actin.

present in crude cell lysate and cytoplasmic or nuclear fractions of VV-infected cells. Fig. 2(*c*) reveals that most of the H5R protein present in crude cell lysate was underphosphorylated (pI 6·8), whereas only a relatively small fraction (about 25%, varying from 17 to 38% in different experiments) was in a highly phosphorylated state (pI 5·5). However, the highly phosphorylated H5R protein was mainly present in the cytoplasmic extract when cell lysates were centrifuged (Fig. 2*d*) and the underphosphorylated H5R protein preferentially sedimented with the nuclei (Fig. 2*e*). PhosphoImager measurements confirmed that the cytoplasmic extracts were considerably enriched in H5R protein with a pI of 5·5 (not shown). It is worthwhile mentioning that substantial amounts of several major acidic viral proteins (designated B, C, D in Fig. 2) also sedimented with the nuclei, whereas another abundant viral protein (designated A) was mainly found in the cytoplasmic fraction. Immunofluorescence observations of VV-infected cells showed that the underphosphorylated H5R protein, specifically recognized by the H5R protein antibody, located

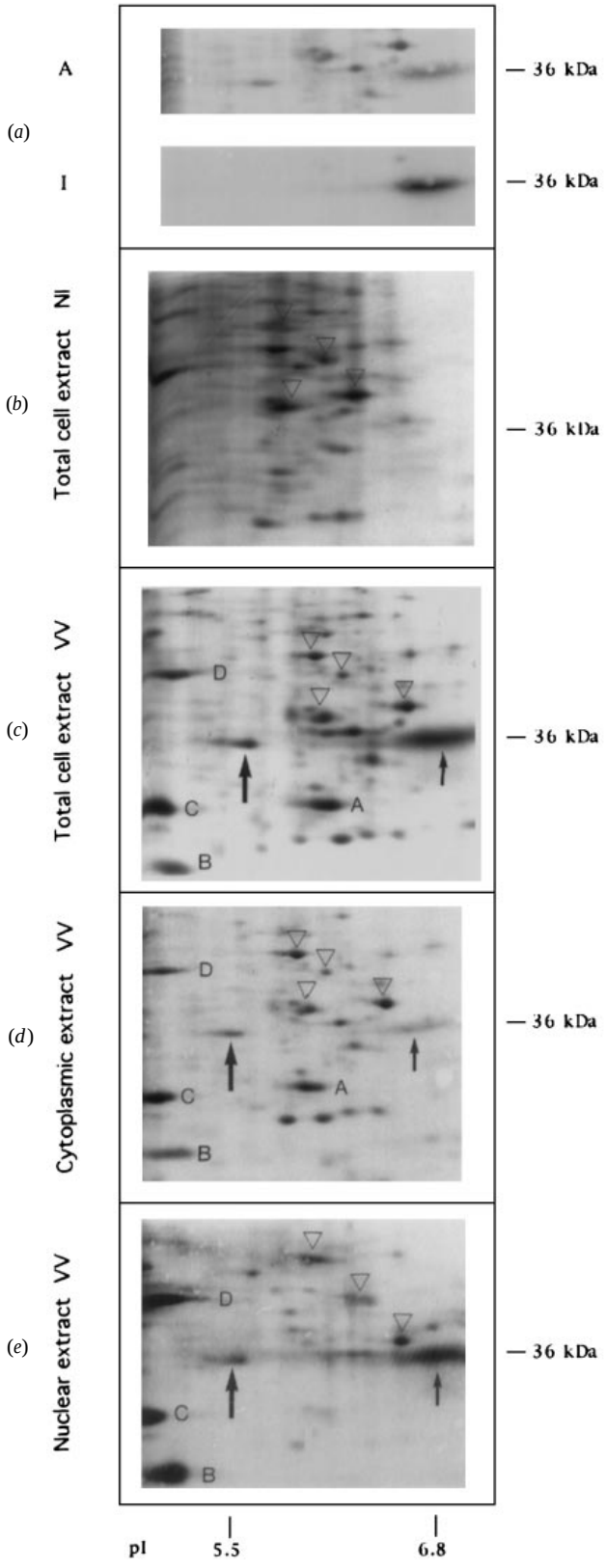

Fig. 2. Phosphorylation state of the H5R protein in BSC-40 cells infected with VV. Cells were infected with WR VV at a ratio of 10 p.f.u. per cell (*a*, *c*, *d*, *e*) or mock-infected (*b*), and then labelled with [^{35}S]methionine and [^{35}S]cysteine for 3·5 h at 37 °C. The cells were washed and total cell lysates (*a*–*c*), cytoplasmic and nuclear extracts were prepared as described in Methods. The relevant part of an immunoblot with H5R protein antibody (I) and corresponding autoradiogram (A) of the blot are shown in (*a*), and autoradiograms of dried gels (*b*, *c*, *e*) or immunoblot (*d*) are shown below. The immunoblot (*d*) is not shown and was similar to that in (*a*). Large arrows indicates the phosphorylated form of the H5R protein (pI 5·5) and smaller arrows indicate the underphosphorylated form (pI 6·8). A, B, C, D in panels (*c*–*e*) indicate viral early proteins and open arrowheads indicate cellular proteins.

to the virosomes, and nuclei were not detectably stained (data not shown). Therefore, it was clear that the granular virosomes, containing mainly underphosphorylated H5R protein, co-sedimented with the nuclei when the cytoplasmic extracts were prepared. Several attempts to separate the nuclei from the granular virosomes by reducing the centrifugation force failed, presumably because the radioactive fractions were prepared in small volumes.

The highly phosphorylated H5R protein (pI 5·5) remained preferentially 'soluble' in the cytoplasmic extract and its cytological location could not be ascertained because it was not recognized by the H5R protein antibody. Consequently, it may be in a truly soluble state or associated with particles or virosomes too small to sediment with the nuclei. The different cytological locations of the highly and the underphosphorylated H5R protein suggest that these forms have different physiological roles. For example, phosphorylation of the H5R protein may be needed to remove the underphosphorylated H5R protein from the part of the virosomal matrix which is committed to be incorporated into virions during assembly. Other roles are of course possible, and it was recently reported that late transcription factor VLTF-4 is encoded by the H5R gene (Kovacs & Moss, 1996).

Because the H5R protein is one of the major early proteins, we investigated the phosphorylation state and cytological location of the H5R protein synthesized in VV-infected cells when viral DNA replication was inhibited by 50 μg/ml 1-(β-D-arabinofuranosyl)cytosine (araC). Two-dimensional gel electrophoresis of total cell lysate and cytoplasmic or nuclear extracts revealed that H5R protein was present only in very low amounts in the nuclear fraction and that the cytoplasmic extract contained a high level of underphosphorylated H5R protein, although the highly phosphorylated form, with a pI of 5·5, was still present (data not shown). Immunofluorescence studies of VV-infected BSC-40 cells in the presence of araC confirmed the biochemical experiments described above because an almost uniform cytoplasmic distribution of underphosphorylated H5R protein was observed, as recently described by Kovacs & Moss (1996). However, we found that some H5R protein was also associated with punctate particles, which were best seen with a confocal microscope and whose number varied in the range of 10–25 particles per cell (Fig. 3*a* and data not shown). In conclusion, these experiments showed that when DNA replication was inhibited, the major part of the H5R protein remained underphosphorylated and was present

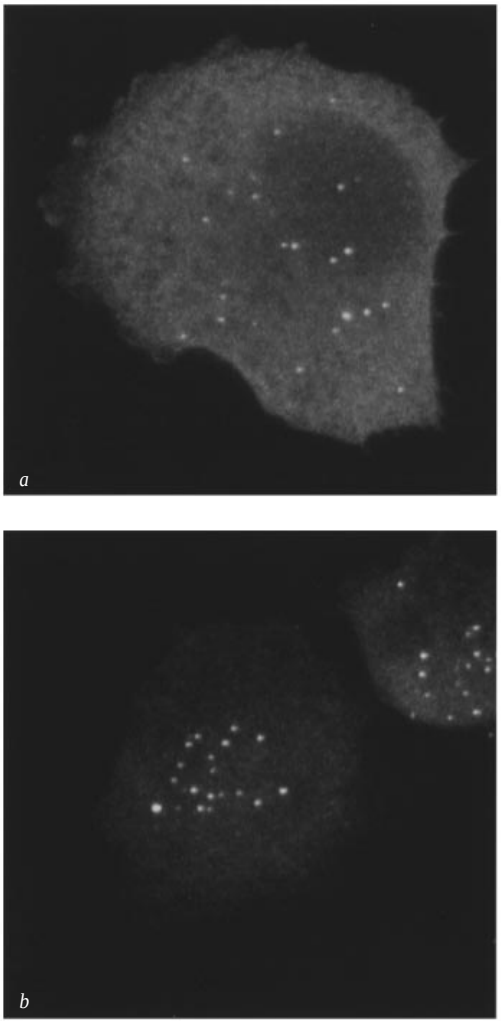

Fig. 3. Uniform and punctate distributions of H5R and B1R proteins by confocal microscopy in cells. BSC-40 cells were infected in the presence of araC for 4 h, fixed with formaldehyde, permeabilized with acetone:methanol (1:1) and processed with either (*a*) the anti-H5R protein serum or (*b*) a B1R antiserum prepared by immunization of a rabbit with the trpE–B1R fusion protein (Banham & Smith, 1992). The second antibody was an FITC conjugate of goat anti-rabbit IgG (Sigma). In each case a typical cell is shown. Confocal laser scanning microscopy was performed using a Leica confocal imaging system (TCS4D) equipped with an argon krypton laser operating with the 488 nm line. Images were collected using an oil immersion lens (60×, NA 1·4 plan Apochromat). A focal series of up to 10 sections apart was collected for each specimen and then processed to produce single composite images (extended focus). Images were printed on dye sublimation printer (Colourease, Kodak).

in the cytoplasm with both a diffuse distribution and a punctate location.

It was thus interesting to investigate the cytological location of the B1R protein synthesized in VV-infected cells when DNA replication was blocked. As described by Banham & Smith (1992), the B1R kinase was mainly located in the virosomes in the case of a permissive infection (data not shown). However, we found that the B1R protein was mainly located at punctate sites in the cytoplasm of cells infected in the presence of araC (Fig. 3 *b*). As in the case of the H5R particles, the number of punctate sites stained by the B1R protein antibody was in the range of 10–25 particles per cell and, in contrast to the H5R protein, only very low levels of B1R protein were uniformly distributed in the cytoplasm (compare Fig. 3 *b* and *a*).

The B1R protein present in the punctate sites was not that known to be associated with the input viral particles (Banham & Smith, 1992; Lin *et al*., 1992) but was synthesized after infection because B1R protein was not detectable by immunofluorescence in cells infected in the presence of 100 μg/ml cycloheximide (data not shown). Furthermore, a kinetics study by confocal microscopy showed that the B1R protein was detectable only after 90 min of infection, began to associate with the particles at 2·5 h after infection and the number of particles formed was at a maximum between 3·5 and 5·5 h (not shown). These kinetics were expected for B1R protein synthesized in the presence of araC (Banham & Smith, 1992). It is tempting to speculate that the newly synthesized B1R protein was targeted towards punctate sites corresponding to DNA replication complexes blocked at the elongation step of viral DNA synthesis. As shown above, we found that underphosphorylated H5R protein also located to similar particles and it is possible that a co-location of the two proteins promotes the phosphorylation of H5R protein by the B1R kinase, but further experiments are needed to clarify the situation and to characterize these particles. Furthermore, it is likely that other early proteins involved in DNA replication do also bind to these particles because a similar punctate location of a p28 virosomal protein, required for ectromelia virus DNA replication in mouse macrophages, was recently described in cells infected in the presence of araC (Senkevich *et al*., 1994, 1995).

Late protein synthesis was not required for binding of the H5R protein to the virosomes formed after reversal of inhibition of DNA synthesis

It was of interest to investigate whether DNA synthesis *per se* is required either for the virosomal location of the H5R protein or to allow the expression of a late (or intermediate) gene which is required for this location. To this end, we infected cells with VV in the presence of 5 mM hydroxyurea (HU) for several hours and then added cycloheximide (to block protein synthesis) before removing the HU and releasing the block on DNA synthesis. At different time intervals, we stained the infected cells with 4′,6-diamidino-2-phenylindole (DAPI) and the H5R protein antibody (specific for the underphosphorylated forms). As expected, virosomes visible by DAPI staining were synthesized when HU was removed from the cell culture medium and, when cycloheximide was added at the time of the HU removal, virosomes were synthesized until 2–3 h after HU removal but their size did not

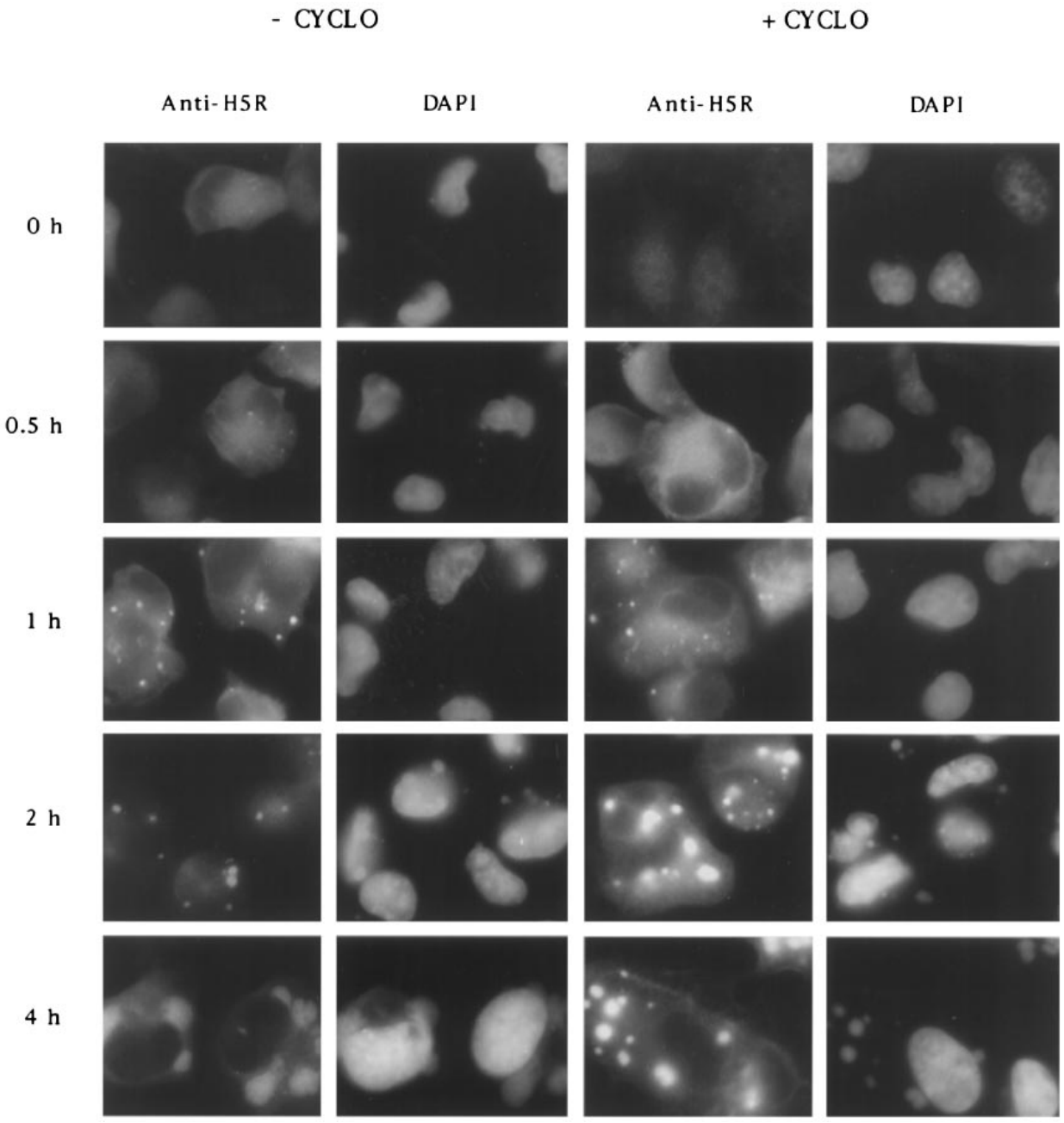

Fig. 4. Binding of the H5R protein to virosomes synthesized in the absence of protein synthesis after reversal of the HU block on DNA synthesis. Cells were infected with WR VV and incubated at 37 °C for 3·5 h in the presence of 5 mM HU. Then, the cell culture medium was removed and the infected cells were washed with PBS and incubated at 37 °C in normal medium without (−cyclo) or containing cycloheximide (+ cyclo). In the latter case, 100 μg/ml cycloheximide was added to cells 15 min before changing the cell culture medium to ensure that protein synthesis was inhibited at the time of HU reversal. At the time indicated in the figure, the cells were processed for immunofluorescence with anti-H5R protein (as described in the legend to Fig. 3) and DAPI staining (1 μg/ml). The same cells were photographed with a Leitz microscope equipped with a mercury lamp and filters to reveal either FITC conjugates (anti-H5R) or DNA (DAPI), except at 2 h (−cyclo), when different fields are shown.

notably increase thereafter (Fig. 4, DAPI) in agreement with previous observations that limited synthesis of viral DNA occurs in these conditions and that viral factories can be formed in the presence of cycloheximide (Esteban & Holowczak, 1978). At the time of HU removal, most of the H5R protein was evenly distributed in the cytoplasm (with punctate location), as expected from the experiments described above and, thereafter, the association of the H5R protein with virosomes occurred in parallel with the synthesis of virosomes (Fig. 4, anti-H5R, −cyclo). When protein synthesis was

blocked at the time of HU removal, the major part of the H5R protein synthesized during the HU block associated with newly synthesized virosomes, as shown in Fig. 4 (anti-H5R protein, + cyclo). It is of note that, although the extent of DNA synthesis was limited, most of the H5R protein synthesized during the HU block did bind to the virosomes subsequently formed in the presence of cycloheximide. Furthermore, two-dimensional gel analyses confirmed that the major part of the H5R protein remained underphosphorylated after reversal of the DNA replication block (data not shown). These experiments demonstrated that the synthesis of late proteins was not required for binding of the H5R protein to virosomal DNA, and suggest direct binding of the H5R protein to newly synthesized DNA, albeit possibly mediated by unidentified cellular or early viral proteins. We have previously shown that the H5R (36 kDa) protein is retained on ssDNA cellulose columns (Beaud *et al.*, 1994, 1995), and therefore the H5R protein may also bind to the ssDNA which is abundant in cells replicating VV DNA (Esteban & Holowczak, 1977; Pogo *et al.*, 1981).

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