Key words: HSV-1/DNA-binding proteins/nuclear matrix

DNA-binding Proteins of Herpes Simplex Virus Type 1-infected BHK Cell Nuclear Matrices

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(Accepted 17 November 1986)

SUMMARY

The nuclear matrix is involved in the replicative cycle of herpes simplex virus type 1 (HSV-1) and in at least some cases viral DNA has been shown to be closely associated with this structure. In this communication, we report the presence of five DNA-binding proteins in the nuclear matrix of HSV-1-infected BHK cells. These proteins (p114, p89, p77, p37 and p29) were detected by probing with \( ^{32} \)P-labelled HSV DNA after Western blotting of nuclear matrix proteins. Three were identified as virion components: p89 as VP12, p77 as VP13 and p37 as the capsid protein VP22a. These polypeptides were detected in cells and nuclei and found to be associated with the nuclear matrix late during the lytic cycle, long after the onset of viral DNA replication. The nuclear matrix-binding capacity of VP22a depended on viral DNA replication, since after DNA polymerase inhibition it was still synthesized and transported into the nucleus but was no longer associated with the nuclear matrix. After inhibition of viral DNA synthesis, VP13 was no longer found in cells, nuclei or nuclear matrices. These results suggest a possible involvement in anchoring viral progeny DNA to the nuclear matrix.

INTRODUCTION

The detection, in recent years, of a nuclear skeleton referred to as the nuclear matrix is a step forward in the understanding of the functional role of the nucleus. There is experimental evidence for possible involvement of the nuclear matrix in DNA replication, transcription and anchoring. The attachment of newly replicated cellular DNA, of a loop structure for interphase DNA and of actively transcribed genes like those for globins and ovalbumin (for review, see Hancock, 1982) illustrates these putative functional and structural roles for the nuclear matrix. Enrichment in viral DNA has been shown upon infection with polyoma virus (Buckler-White et al., 1980), adenovirus (Younghusband & Maundrell, 1982) and pseudorabies virus (Ben-Porat et al., 1984). There are indications that this structure is involved in herpes simplex virus type 1 (HSV-1) virogenesis: electron micrographs of infected BHK cell nuclear matrices show an accumulation of viral capsids (Bibor-Hardy et al., 1982), many infected cell polypeptides (ICPs) can be detected by SDS-PAGE analysis (Bibor-Hardy et al., 1985a, b), detection of HSV-1 DNA polymerase activity in nuclear membranes has been reported (Herzberg et al., 1981) and newly replicated HSV-1 viral DNA is attached on the nuclear matrix (D. Villard et al., unpublished results).

All these facts have led us to look for DNA-binding proteins in the nuclear matrix of HSV-1-infected cells. Among the numerous HSV-specified ICPs, at least 17 proteins have DNA-binding properties, as monitored by DNA–cellulose affinity chromatography (Bayliss et al., 1975; Powell & Purifoy, 1976; Becker et al., 1980). At least two of these, the major DNA-binding protein (Littler et al., 1983) and the viral DNA polymerase (Powell & Purifoy, 1977), have been well characterized. But little is known about the precise functions of most of the DNA-binding proteins; they could take part in any of the intricate mechanisms underlying viral DNA replication, processing or packaging.
A DNA-binding assay, involving protein transfer from polyacrylamide gels to nitrocellulose paper (Western blotting) and probing of the blots of the $^{32}$P-labelled DNA, has been described by Russell & Precious (1982). This communication reports the use of this technique, of particular interest for highly insoluble structures like nuclear skeletons, to investigate the presence of cellular and viral DNA-binding proteins in HSV-1-infected BHK cell nuclear matrices.

**METHODS**

**Cell culture and infection.** In all these experiments, BHK-21 cells (clone 13) were grown in α-medium supplemented with 10% foetal calf serum. For the preparation of nuclear matrices, confluent cells were infected with HSV-1 tsG8 strain KOS (from Dr P. Schaffer) as previously described (Bibor-Hardy et al., 1985a), using a m.o.i. of 5 p.f.u. per cell. Infected cells were harvested after 16 h at 34 °C by scraping off into the medium with a rubber policeman.

**Nuclear matrix protein preparation.** Nuclear matrices were isolated from normal or infected cells, using a modified version of the method of Berezney & Coffey (1977) (described in Dagenais et al., 1984). Briefly, the harvested cells were washed twice in phosphate-buffered saline and incubated 10 min on ice in a hypotonic TECK buffer (10 mM-Tris-HCl pH 7.8, 3 mM-CaCl$_2$, 1 mM-EDTA, 10 mM-KCl). Clean nuclei were prepared using an Ultra-Turrax (Janke & Kunkel), and washed twice in the same buffer. To obtain nuclear matrices, the purified nuclei were then successively treated twice with a low salt buffer (10 mM-Tris–HCl pH 7.4, 0.2 mM-MgCl$_2$), digested with DNase I (10 μg/ml; at room temperature for 20 min) and extracted twice in a high salt buffer (10 mM-Tris–HCl pH 7.4, 0.2 M-MgCl$_2$, 2 M-NaCl). Treatment with Triton X-100 was omitted in order to isolate nuclear matrices with intact nuclear membranes. SDS–PAGE protein analysis of cells, nuclei and matrices were performed as described elsewhere (Bibor-Hardy et al., 1985a) using a 5 to 12% polyacrylamide gradient.

**Purification of HSV virions.** Enveloped virus particles were purified from cytoplasmic extracts of HSV-1-infected cells, essentially as described by Spear & Roizman (1972). BHK cells were infected at 1 p.f.u. per cell and incubated 36 h at 34 °C. The infected cell pellet was resuspended in a small volume of TECK buffer and disrupted as described for the preparation of clean nuclei. The nuclei were then centrifuged and the cytoplasmic extract was centrifuged through a 35 ml dextran T 10 density gradient (1.04 to 1.09 g/ml) for 1 h at 23000 r.p.m. in a Beckman SW27 rotor. The virions found in the middle of the gradient were pelleted by centrifugation at 25000 r.p.m. for 1 h (SW27 rotor).

**DNA–protein association.** The DNA-binding assay was done according to Russell & Precious (1982). The electrophoretically separated proteins were transferred to nitrocellulose and renatured for 1 h at room temperature in binding buffer (10 mM-Tris–HCl pH 7.4, 50 mM-NaCl, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone) containing 2.5% NP40 to remove SDS. After renaturation, the nitrocellulose paper was washed 30 min in binding buffer without NP40. Most of the blots were incubated for 30 min with unlabelled salmon sperm DNA (100 μg/ml) prior to the addition of $^{32}$P-DNA. The DNA binding assay was started by adding nick-translated $^{32}$P-labelled total HSV-1 DNA ($2 \times 10^6$ c.p.m./ml) to the blots. After 4 h incubation, the blots were carefully washed and allowed to dry before exposure to Kodak X AR film with an intensifying screen.

**RESULTS**

**Detection of matrix-associated DNA-binding proteins**

Coomassie Brilliant Blue staining revealed the presence of numerous polypeptide bands in cellular, nuclear and even in the nuclear matrix samples. Since all the proteins from nuclear membranes were still present, the only major visible difference between nuclei and nuclear matrices seemed to be the diminution or absence of histones in the matrix (Fig. 1a, lanes 3 and 5). More than 20 DNA-binding proteins were detected in uninfected cells; in addition to the bands already present, new ones appeared after HSV-1 infection (Fig. 1b, lanes 1 and 2). The same pattern was observed for nuclei (Fig. 1b, lanes 3 and 4). However, after extraction with low salt buffer and treatment with DNase and 2 M-NaCl, only a small subset of the DNA-binding proteins remained in the nuclear membranes (Fig. 1b, lanes 5 and 6). There were significant differences in the protein content of the infected cell matrix: polypeptides p114, p77 and p37 were found only after infection, and five of the six proteins originally present in the uninfected matrix disappeared. Protein p60 was present in both infected and uninfected nuclear matrices. All these proteins were minor components of the nuclear matrices, since they were barely visible on Coomassie Brilliant Blue-stained gels (Fig. 1a, lanes 5 and 6). Among the three virus-induced proteins, p37 gave the strongest signal; p114 seemed to be labile since the intensity of the band varied among preparations.

Incubation of the blots with unlabelled salmon sperm DNA (100 μg/ml) prior to the addition
Fig. 1. Association of DNA-binding proteins with normal or HSV-1-infected nuclear matrices. From cells (lanes 1 and 2), nuclei (lanes 3 and 4) or nuclear matrices (lanes 5 and 6), equal amounts of protein (50 µg) were loaded on each lane for SDS-PAGE analysis. After electrophoresis, the proteins were either revealed by Coomassie Brilliant Blue staining (a), or transferred to nitrocellulose paper and probed directly with 32P-labelled HSV-1 DNA (b) or transferred to nitrocellulose and incubated with salmon sperm DNA (100 µg/ml) before probing with 32P-labelled HSV-1 DNA (c). Odd-numbered lanes represent uninfected samples and even-numbered lanes infected extracts. The autoradiograms were exposed for 16 h (b) or 3 days (c). Mol. wt. is indicated × 10^{-3}.

of 32P-labelled HSV-1 DNA reduced the signal given by many cellular proteins (Fig. 1 c). In the infected nuclear matrix, p60 was no longer detectable and two additional DNA-binding proteins were seen: p89 and p29, in addition to p77 and p37 (Fig. 1 c, lane 6). Sometimes, a faint trace of p114 could still be observed. Under these conditions, the DNA-binding signal increased in
Fig. 2. Detection level for the HSV-1 DNA-binding proteins. Increasing amounts of HSV-1-infected nuclear matrix proteins were separated by SDS-PAGE; lanes 1 to 4: 5, 10, 20 and 40 μg. After electrophoresis the proteins were transferred to nitrocellulose, incubated with salmon sperm DNA and probed with $^{32}$P-labelled HSV-1 DNA. The autoradiogram was exposed for 4 days.

proportion to the amount of protein loaded on the gel (Fig. 2). Incubation with a tenfold higher concentration of salmon sperm DNA did not change the previously observed pattern shown in Fig. 1(c) (results not shown).

**Identification of the DNA-binding proteins**

To find out whether any of the five DNA-binding proteins were virion proteins, purified HSV-1 virion proteins were probed with $^{32}$P-labelled HSV-1 viral DNA (Fig. 3a, b, lanes 2). Of the 15 viral polypeptides clearly detected by Coomassie Brilliant Blue staining (Fig. 3, lane 3), only four showed affinity for total HSV-1 DNA: VP12, VP13, VP14 and VP22. Two of these proteins comigrated with nuclear matrix-associated DNA-binding proteins (Fig. 3a, b, lanes 1 and 2): VP12 with p89 and VP13 with p77. p37 has a slightly higher mol. wt. than VP22 and may correspond to VP22a identified in type B viral capsids isolated from the nucleus (Gibson & Roizman, 1972).

**Infection kinetics**

The experiments described above demonstrated that only five virus-induced DNA-binding proteins were associated with the nuclear matrix 16 h after HSV-1 infection. We next examined at which stage of infection these proteins became associated with the nuclear matrix, and whether different DNA-binding proteins were responsible for viral DNA attachment to this structure at earlier times of infection. For this purpose, nuclear matrices were isolated at different times after infection (0, 4, 8, 12 and 16 h) and equal amounts of protein from cells or matrices were loaded on SDS-polyacrylamide gels for separation and incubation with labelled DNA as described above. There was no difference between the DNA-binding protein patterns of normal and infected cells up to 4 h (Fig. 4, lanes 1 and 2). At later stages, additional bands corresponding to p89, p77 and p37 appeared in the infected cell (Fig. 4, lanes 3, 4 and 5). All the DNA-binding proteins found in association with the infected nuclear matrix, p89, p77, p37 and p29, appeared late in infection, long after the onset of viral DNA replication (Fig. 4, lanes 8, 9 and 10). They were first detected in the cell samples and there was a delay before they became
Fig. 3. Identification of the proteins. Equal amounts of protein (50 μg) from HSV-1-infected nuclear matrix (lanes 1) or HSV-1 virions (lanes 2 and 3) were separated by SDS-PAGE, and probed for their HSV-1 DNA-binding properties in the presence of salmon sperm DNA. The autoradiogram was exposed for 1 day (a) or 1 h (b). After electrophoresis, the proteins of the HSV-1 virion (VP) were also revealed by Coomassie Brilliant Blue staining (lane 3).

associated with the matrix. Interestingly, cellular DNA-binding proteins were almost absent from nuclear matrices at 8 h (Fig. 4, lane 8), although they were still present in the cells (Fig. 4, lane 3).

To determine whether viral DNA synthesis was necessary for the association of the DNA-binding proteins with the nuclear matrix, phosphonoformate (PFA), an inhibitor of viral DNA polymerase, was added to the culture medium during infection at concentrations varying from 100 to 400 μg/ml. Nuclear matrix-associated DNA-binding proteins p114, p89 (VP12), p77 (VP13) and p29 were affected by PFA treatment; they decreased with increasing quantities of inhibitor (Fig. 5c, lanes 2, 3 and 4). The same effect was clearly observed in cells and nuclei for p77 (VP13) indicating a decrease in its synthesis and/or activity upon PFA treatment.

However, p37 (VP22a) behaved differently: it decreased in nuclear matrices upon PFA treatment but remained constant in cells and nuclei (Fig. 5). This observation implies that the association of p37 (VP22a) with the matrix depended on DNA replication but that its synthesis and transport to the nucleus were not affected by the treatment.

**DISCUSSION**

The nuclear matrix has been implicated in many aspects of herpes virogenesis. A role in capsid assembly now seems a real possibility, since polypeptide profiles and electron
Fig. 4. Kinetics of appearance of nuclear matrix-associated DNA-binding proteins. Proteins from (a) cells or (b) nuclear matrices were assayed for their DNA-binding properties with ³²P-labelled HSV-1 DNA in the presence of salmon sperm DNA at different times post-infection. Lanes 1 and 6, 0 h; lanes 2 and 7, 4 h; lanes 3 and 8, 8 h; lanes 4 and 9, 12 h; lanes 5 and 10, 16 h. The autoradiogram was exposed for 4 days.

Fig. 5. Effect of PFA on nuclear matrix-associated DNA-binding proteins. Different concentrations of PFA were added to infected cells after virus adsorption. Proteins from (a) 16 h infected cells, (b) nuclei or (c) nuclear matrix were assayed for their DNA-binding properties with ³²P-labelled HSV-1 DNA in the presence of salmon sperm DNA. PFA concentrations were (1) 0 µg/ml, (2) 100 µg/ml, (3) 200 µg/ml or (4) 400 µg/ml. The autoradiograph was exposed for 4 days.
micrographs suggest that DNA-deficient type A capsids (Gibson & Roizman, 1972) are enriched in matrix preparations (Bibor-Hardy et al., 1982; Tsutsui et al., 1983), and the major capsid protein ICP5 is preferentially retained in this structure (Bibor-Hardy et al., 1985b). A role in viral DNA replication has been suggested by Ben-Porat et al. (1984) who found attachment of replicating forms of DNA but failed to demonstrate preferential retention of specific capsid proteins in the matrix of cells infected by pseudorabies virus. We also have found that HSV-1 DNA is attached to the nuclear matrix at all stages of the lytic cycle. No specific sequences seem to be responsible for this attachment (D. Villard et al., unpublished results). To explain the retention of viral DNA, one possibility is that proteins associated with the nuclear matrix are able to anchor viral DNA.

We have used an assay involving DNA binding on nitrocellulose filters to detect DNA-binding proteins in HSV-1-infected nuclear matrices. This technique, although of great interest for the study of insoluble structures such as the cytoskeleton or nuclear matrix, nevertheless has limitations. A comparison of our results with experimental data obtained elsewhere shows that, for example, the major HSV-1 DNA-binding protein (ICP8 or 130K) was easily detected on DNA-cellulose affinity columns (Powell & Purifoy, 1976) but escaped our binding assay in infected cells and nuclei (Fig. 1 b or c, lanes 2 and 4). This may be due to the use of SDS and 2-mercaptoethanol in our system since these substances can destroy the binding properties of some proteins, among which is probably ICP8.

Many DNA-binding proteins are detected in our normal or infected nuclear matrix preparations. However, in the presence of a 4000-fold excess of salmon sperm DNA only four proteins migrating at 89K, 77K, 37K and 29K are able to bind total HSV-1 DNA. These proteins are present in very small amounts in our matrix preparations. Although not detectable by Coomassie Brilliant Blue staining of nuclear matrix proteins, they appear with striking clarity after the DNA-binding assay. This technique is therefore very useful for the identification of minute quantities of otherwise undetectable DNA-binding proteins.

Since the infected nuclear matrix contained numerous viral capsids, we have looked at the DNA-binding properties of the virus proteins. The isolated HSV-1 virion, fully enveloped, contains 24 to 33 proteins (Heine et al., 1974); of these, only four, VP12, VP13, VP14 and VP22 are able to bind HSV-1 DNA under our conditions. Blair & Honess (1983) have obtained similar results with HSV-1 virions probed with herpesvirus saimiri DNA. They found DNA-binding proteins in the VP11 to VP14 region and in the VP22 to VP23 region. Braun et al. (1984) have used the blotting technique to analyse the DNA-binding proteins of nuclear and cytoplasmic viral capsids and described only one protein of 55K (VP 19C) with affinity for DNA. Neither in the total virion nor in the infected matrix did we detect any major DNA-binding polypeptide in the 50K to 55K mol. wt. range. This discrepancy should be investigated further.

Only a few virion polypeptides have been investigated with respect to their function. Pignatti & Cassai (1980) have isolated a nucleoprotein complex devoid of DNA-replicating and -transcribing activity, and reported the presence of VP12 and traces of VP13 and VP14 in these complexes along with viral DNA and a few other viral proteins. They first appear 9 h post-infection and increase in quantity up to 18 h. Although the significance of these nucleoprotein complexes is not clear, Pignatti & Cassai proposed that they represent an intermediate in virion assembly. The nuclear matrix DNA-binding proteins p89 and p77 comigrate with VP12 and VP13 of HSV-1. Even if VP14 is a DNA-binding protein, it is not associated with the nuclear matrix. The time course of association with the nuclear matrix for p89 (VP12) and p77 (VP13) closely parallels that of HSV nucleocomplex formation and these two proteins may be responsible for the retention of viral DNA in the nuclear matrix prior to encapsidation, at these late times of infection.

The precursor of VP22, VP22a, has been identified by Gibson & Roizman (1972) in the HSV-1 system as part of the nucleocapsid polypeptide complex in type B capsids which contain 10 times more DNA than type A capsids. Since then, various investigators have described similar proteins in HSV type 2 (Zweig et al., 1980), pseudorabies virus (Ladin et al., 1980, 1982) and cytomegalovirus (Gibson, 1981). Furthermore, the analogous polypeptide in varicella-zoster virus-infected cells, p32/p36, has been found in association with the nuclear matrix (Friedrichs...
& Grose, 1986). Evidence obtained using a temperature-sensitive mutant suggests that VP22a is necessary for DNA insertion and encapsidation (Preston et al., 1983). Our finding that p37 (VP22a) has DNA-binding capacity and is associated with the nuclear matrix correlates well with these results. The synthesis and transport of p37 to the nucleus are clearly not dependent upon viral DNA synthesis. Although it is not known whether the synthesis of VP22a is affected by PFA, a behaviour similar to that of p37 described here was observed using herpesvirus saimiri. In this report also, a 29K DNA-binding protein was found associated with capsids (Blair & Honess, 1983), and was processed from a 31K phosphonoacetic acid-resistant precursor in the nucleus of the infected cell. To explain the lack of association with the nuclear matrix upon PFA treatment, we postulate that p37 (VP22a) forms a complex with the capsid itself. In this model, the association of p37 (VP22a) with the nuclear matrix is mediated by the attachment of viral capsid to the matrix.

Purification of the five nuclear matrix-associated DNA-binding proteins is under way to determine their main properties. These proteins appear late upon infection, and therefore although their presence in the nuclear matrix can explain the attachment of viral progeny DNA to the matrix, the question of how newly replicated viral DNA is attached to the nuclear matrix is still unanswered.

We thank Benoit Coulombe for his useful suggestions. We thank also André LeMyre and Eve St-Pierre for their excellent technical assistance. This work was supported by grants from the NCI and MRC of Canada. M.-F.P. is supported by a studentship from the MRC.

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(Received 12 March 1986)