DNA-binding Proteins Induced by the Cottontail Rabbit Herpesvirus CTHV

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(Accepted 2 June 1988)

SUMMARY

Several new polypeptides were detected in cells infected with CTHV, a cottontail rabbit herpesvirus. All of them (Mr 150K, 110K, 93K, 83K, 75K and 35K) accumulated in the nucleus during the infectious cycle, and all except the 150K species bound to DNA-cellulose affinity columns in low-salt buffers. Polyclonal antisera prepared against the 35K DNA-binding protein also recognized the 75K species. Although the 75K protein could be detected earlier in infection than the 35K protein, late in the infectious cycle the latter increased to an abundance approaching that of cellular histones. Treatment of partially purified virions with a non-ionic detergent indicated that the 35K protein, but not the 75K protein, is a component of capsid/tegument structures. The anti-35K/75K serum did not cross-react with herpesvirus sylvilagus virion proteins, which, in an electrophoretic comparison, exhibited both similarities to and differences from the virion proteins of CTHV. Labelling of CTHV-infected cells with [32p]orthophosphate revealed the presence of phosphoproteins electrophoretically comigrating with the 93K, 83K, 75K and 35K proteins.

INTRODUCTION

Herpesvirus genomes, all of which are linear duplex DNA molecules, exist in a variety of sizes, base compositions and sequence arrangements (for a recent review, see Honess, 1984). Amid this variety, one structural pattern is especially remarkable in that it embodies the extremes of base composition and sequence repetition currently recognized for the herpesvirus group as a whole. Originally discovered in herpesvirus saimiri (Bornkamm et al., 1976), similar genome structures have since been found in other New World primate herpesviruses (reviewed by Fleckenstein & Desrosiers, 1982), several bovine herpesviruses (Ehlers et al., 1985), and two independently isolated cottontail rabbit herpesviruses, herpesvirus sylvilagus (Medveczky et al., 1984; see, however, Rouhandeh & Cohrs, 1987) and CTHV (J. Cebrian et al., personal communication).

In this structural pattern roughly one-third of the 150 kilobase pair (kbp) genome is given over to tandem repeats of a 0-9 to 2-7 kbp sequence with a very high (70 to 75%) G + C content. The remainder of the genome consists of a continuous stretch of (coding) sequences with a very low (30 to 35%) G + C content. Clusters of variable lengths of the G + C-rich repeats are appended with direct polarity to both ends of the G + C-poor region. In a variation found in CTHV, the G + C-poor region is interrupted by an inverted variable length cluster of the same repeats present in the terminal clusters. The variability in length of the repeat clusters is complementary, in that a change in one cluster is accompanied by a compensating change in another. Thus, the total number of repeat units in the genome remains constant. The complementarity is most easily understood in terms of a model in which unit-length genomes are cut from concatemers during the encapsidation of viral DNA, and that this process is mediated by sequences in the G+C-rich repeat unit (Bankier et al., 1985). Since this recognition process is expected to require specific protein–DNA interactions, a description of DNA-binding proteins (DBPs) present during infection is of interest.
We have undertaken a study of DBPs in CTHV-infected cells. We chose CTHV to provide comparative data for the viruses of this group, since herpesvirus saimiri has already been the object of a similar study (Blair & Honess, 1983), and descriptions have appeared of virus-specific proteins induced by infection with herpesvirus sylvilagus (Cohrs & Rouhandeh, 1982; Patick & Hinze, 1984; Rouhandeh & Cohrs, 1984). The hope is that such comparisons may eventually shed light on how these peculiar genome structures originated and how they are maintained.

METHODS

**Cells and virus.** The continuous rabbit kidney cell line RK13 (obtained from the American Type Culture Collection) was used for large scale culture and titration of virus. CTHV was isolated from a primary culture of cottontail rabbit (Sylvilagus floridanus) kidney cells and kindly provided by Dr G. Orth (Pasteur Institute, Paris, France). Herpesvirus sylvilagus was a generous gift of Dr C. Mulder (University of Massachusetts, Worcester, Mass., U.S.A.). Cells were propagated at 37 °C in Eagle’s minimal essential medium (MEM) supplemented with 10% foetal calf serum (FCS). Virus infection was performed under the same conditions except that the concentration of FCS was 2%. Plaque titration of infectious virus was carried out on cell monolayers in 30 mm plastic Petri dishes in MEM–2% FCS, supplemented with 0.6% Bacto-Agar (Difco). After 6 days at 37 °C, monolayers were incubated with 0.1% neutral red in phosphate-buffered saline (PBS) and plaques were counted.

To prepare purified virions, supernatants of infected (for 5 days) cultures (0.1 p.f.u. per cell at 37 °C) were centrifuged at 16000 g for 2 h and the resuspended pellets (in PBS) sedimented through 10 to 40% (w/w) sucrose gradients buffered with 200 mM-NaCl, 20 mM-sodium phosphate pH 7.5, 2 mM-EDTA in an SW41 rotor at 17000 r.p.m. at 4 °C for 40 min. Light-scattering bands near the middle of the gradients were collected, diluted 20-fold with PBS, the virions were concentrated by centrifugation as above and resuspension of the pellets was in 200 mM-NaCl, 20 mM-sodium phosphate pH 7.5, 2 mM-EDTA. Capsid/tegument structures and soluble envelope proteins were generated by incubation at room temperature for 30 min in 0.5% Nonidet P40 (NP40) and subsequent centrifugation at 13000 g for 2 h to obtain pellet and supernatant fractions.

**Radioactive labelling of infected and mock-infected cells.** Subconfluent RK13 cell monolayers in 60 mm (1 × 10^6 cells) or 100 mm (5 × 10^6 cells) plastic Petri dishes were infected with CTHV at a multiplicity of 10 p.f.u. per cell. Adsorption was in MEM–2% FCS at 37 °C for 1 h. Mock infection was done with the same volume of medium lacking virus. At the appropriate time, the medium was removed and the monolayer rinsed with MEM–2% FCS containing 10% of the standard concentration of methionine or orthophosphate, depending on the label to be used. Incubation was then continued in the medium supplemented with 10 μCi/ml of [35S]s-methionine (1000 Ci/mmol; New England Nuclear) or [32P]orthophosphate (10 μCi/ml; Commission de l’Energie Atomique, Saclay, France).

**Preparation of cell extracts.** CTHV-infected or mock-infected cell monolayers in plastic Petri dishes were harvested by scraping into the medium and were then centrifuged at 1600 g for 10 min at 4 °C. The cell pellet was washed once with PBS and resuspended for 10 min at 4 °C in RBS buffer (10 mM-NaCl, 10 mM-Tris–HCl pH 7.4, 1.5 mM-MgCl2) and 1% NP40, at a concentration of 10^7 cells per ml. The cells were disrupted with a Dounce homogenizer (usually 10 to 20 strokes), nuclei were pelleted at 1000 g for 10 min at 4 °C, and the supernatant cytoplasmic extract was stored at −70 °C until use. The nuclear pellet was resuspended in 20 mM-Tris–HCl pH 7.4, 5 mM-EDTA, 2 mM-mercaptoethanol, 2 mM-PMSF, 10% glycerol (buffer A) containing 150 mM-NaCl, at a concentration of 5 × 10^6 nuclei/ml and lysed by the addition of NaCl to a final concentration of 2 M. Following brief sonication (Branson model J-22) to reduce viscosity, the lysate was held at 0 °C for 1 h, clarified by centrifugation at 140 000 g for 1 h at 4 °C, and dialysed against several changes of buffer A adjusted to 50 mM-NaCl. Protein concentration was determined by the method of Schaffner & Weissmann (1973).

In some experiments, DNA–histone complexes were precipitated from nuclear lysates with spermine as described by Blair & Honess (1983), but since no major differences, with the exception of histones, were observed in the number or quantity of DBPs, this step was omitted in most preparations.

**SDS–PAGE.** Samples were heated at 100 °C for 10 min in SDS–2-mercaptoethanol dissociation buffer and electrophoresed through 210 mm × 150 mm slab gels consisting of a 15% acrylamide running phase and a 5% acrylamide stacking phase as described by Laemmli (1970). For direct detection of polypeptides, gels were stained with 0.25% Coomassie Brilliant Blue in 50% methanol–10% acetic acid, destained in 20% methanol–5% acetic acid and dried under vacuum. Autoradiography was carried out with Fuji RX X-ray film.

**Filter binding assay.** Nitrocellulose discs (BA85, 25 mm diameter; Schleicher & Schüll) were boiled in binding buffer (50 mM-NaCl, 10 mM-Tris–HCl pH 7.5, 10 mM-magnesium acetate, 1 mM-EDTA, 5 mM-2-mercaptoethanol) before use. Various amounts of dialysed nuclear extract were incubated in binding buffer (50 μl final volume) with 5 ng 32P-labelled pBR327 DNA (2 × 10^6 c.p.m./μg) for 30 min at room temperature. After passage of the incubation mixture at low vacuum, the filters were washed with four 0.5 ml portions of binding buffer and dried, and retained radioactivity was determined by scintillation counting.
DNA–cellulose affinity chromatography. Samples of dialysed nuclear extract containing 2 to 5 mg protein (from 1.2 x 10⁶ cells) were applied to columns of calf thymus dsDNA linked to cellulose (5.7 mg DNA/g cellulose; Sigma) with a bed volume of 2-5 ml (approx. 2 to 5 mg DNA) and washed thoroughly with buffer A containing 50 mM-NaCl. All manipulations were carried out at 4 °C. Bound proteins were eluted with a 15 ml gradient of 50 mM- to 1.5 M-NaCl in buffer A at a flow rate of 10 ml/h. Aliquots of collected fractions were taken for the determination of radioactivity by liquid scintillation counting or for SDS–PAGE and autoradiography.

Preparation of anti-35K DBP serum. Peak fractions from a DNA affinity column were combined (30 μg total protein) and subjected to SDS–PAGE. Regions of the gel containing the M, 35K species were excised, fragmented with a Dounce homogenizer, and inoculated into New Zealand rabbits by the subcutaneous route. Following three injections at intervals of 15 days, positive reactions were generally obtained in immunoblot assays. Three independent antisera gave similar results.

Immunoblotting procedures. Proteins were transferred from gels to nitrocellulose sheets (BA83; Schleicher & Schüll) as described by Towbin et al. (1979). Loaded sheets were preincubated for at least 2 h at room temperature with 0.5% Tween 20 (Sigma) in PBS. The primary antiserum was then added to a final dilution of 1/1000 and incubation was continued overnight under gentle agitation at 4 °C. After thorough washing with PBS, peroxidase-conjugated goat anti-rabbit IgG antiserum (Diagnostics Pasteur) diluted 1/500 in PBS was added; incubation was for 3 h at room temperature. The substrate for colour development was 4-chloro-1-naphthol.

For affinity purification of anti-35K or anti-75K antibodies, [³⁵S]methionine-labelled proteins in DNA–cellulose column eluates were subjected to SDS–PAGE, transferred to nitrocellulose as above, and located by autoradiography. Patches were cut from regions corresponding to the 35K or 75K bands and from intermediate regions as controls. The patches were first incubated with antiserum and washed as above, then eluted in acid buffer as described by Smith & Fisher (1984).

RESULTS

Single cycle growth: production of infectious virus and appearance of virus-induced polypeptides

CTHV grows slowly in cultured RK13 cells. As shown in Fig. 1, under single cycle growth conditions, maximum yields of infectious virus (approx. 50 p.f.u. per infected cell) were obtained only after 72 h of incubation at 37 °C. Although we have not exhaustively investigated various parameters that might influence optimal growth, the use of various cell types, media, serum concentrations or incubation temperatures did not markedly shorten the growth cycle (unpublished data). The slow growth of CTHV resembles the growth properties of herpesvirus sylvilagus (Patick & Hinze, 1984) and herpesvirus saimiri (Modrow & Wolf, 1983; Randall et al., 1983) in tissue culture.

![Fig. 1. Single-cycle growth curve of CTHV in RK13 cells. Cells were infected at 10 p.f.u. per cell. At the appropriate times, cultures were frozen and titrated as described in Methods.](image)
Fig. 2. Electrophoretic profiles of labelled protein extracts from CTHV-infected and mock-infected RK13 cells. [35S]Methionine was added to cultures 24 h before harvesting at (a) 72 h or (b) different times up to 72 h. Nuclear and cytoplasmic extracts were prepared, subjected to SDS-PAGE, and autoradiographed as described in Methods. (a) Lanes 1 and 3, cytoplasmic extracts of mock-infected and infected cells respectively; lanes 2 and 4, nuclear extracts of mock-infected and infected cells respectively. (b) Nuclear extracts of: lane 1, cells 72 h after mock infection; lanes 2 to 4, infected cells 24 h, 48 h and 72 h p.i. respectively. Positions of Mr, marker proteins are indicated on each side of the figure (β-galactosidase, 116K; phosphorylase b, 94K; bovine serum albumin, 67K; ovalbumin, 43K; carbonic anhydrase, 30K; soybean trypsin inhibitor, 20K).

In order to examine the synthesis of polypeptides specific to CTHV infection, cultures were labelled with [35S]methionine at various times after infection and analysed by SDS-PAGE. Preliminary experiments revealed that even at high m.o.i. a substantial level of host protein synthesis throughout the infectious cycle made it difficult to detect virus-specific polypeptides in
total cell extracts (not shown). Subsequently, an examination of subcellular fractions permitted the detection of several virus-induced polypeptides accumulating in the nucleus (Fig. 2). The electrophoretic patterns of cytoplasmic and nuclear extracts of mock-infected and infected cells harvested at 72 h post-infection (p.i.) are presented in Fig. 2(a). The profiles of cytoplasmic extracts showed only minor differences, and the vast majority of the $[^{35}\text{S}]$methionine-labelled proteins present in CTHV-infected cells were also found in mock-infected cells (lanes 1 and 3). Two exceptions were faint bands at positions corresponding to $M_t$ values of 150K and 35K in the profile of infected cells.

In contrast, six new polypeptide species were readily apparent in the corresponding nuclear extracts of infected cells (Fig 2a, lane 4). The relative mobilities of these indicate $M_t$ values of approximately 150K, 110K, 93K, 83K, 75K and 35K. For as yet undetermined reasons, the relative intensities of the various bands were subject to moderate fluctuation between experiments (compare 72 h p.i. extracts in Fig. 2a and b), although in most cases the 35K polypeptide was the major species (see below). The time course of appearance for the set of nuclear polypeptides is shown in Fig. 2 (b). All six became detectable by 48 h p.i., and the 93K, 83K and 75K polypeptides had already attained their maximum levels at this time. By 72 h p.i. the levels of the remaining three polypeptides had undergone moderate (150K and 110K) to substantial (35K) increases.

Detection of DBPs in nuclear extracts of infected cells

Preliminary examination of DNA binding by means of nitrocellulose filter binding assays revealed considerably enhanced activity in nuclear extracts of CTHV-infected cells. Titration curves for extracts of mock-infected and 72 h p.i. CTHV-infected nuclei are shown in Fig. 3. Extracts of infected nuclei were typically capable of retaining 70% to 95% of the input DNA, depending on the experiment, while at equivalent protein concentrations less than 5% of the input was bound by mock-infected extracts.

In order to determine which nuclear proteins were capable of binding DNA, affinity columns of native or denatured calf thymus DNA were prepared. Chromatography of $[^{35}\text{S}]$-labelled nuclear extracts from 72 h p.i. CTHV-infected cells on a native DNA column is presented in Fig. 4. Gradient elution of column-bound material resulted in a tailed peak with a maximum centred at approximately 0.45 M-NaCl (Fig. 4a). In this and numerous other experiments, 20% to 40% of the input $[^{35}\text{S}]$methionine was retained by the column; decreasing the amount of protein applied to the column did not increase the percentage retained. Under the same chromatographic
Fig. 4. DNA affinity chromatography of 72 h p.i. nuclear proteins. [35S]Methionine-labelled nuclear extracts were applied to a column of native calf thymus DNA-cellulose and eluted as described in Methods. Fractions were subjected to SDS-PAGE, stained and autoradiographed. (a) Radioactivity in eluted fractions (●); NaCl concentration (○). (b) Autoradiogram following SDS-PAGE of selected fractions. (c) Coomassie Brilliant Blue stain of gel in (b). Fraction numbers are indicated at the top of the gel in (b) and (c); N represents total nuclear extract (unfractionated). $M_r$ values are indicated.
Fig. 5. Immunoblotting with a polyclonal anti-35K/75K serum. Nitrocellulose blots of mock-infected cell nuclear extracts (lanes 3 and 6) and pooled fractions 24 to 26 from column of Fig. 4 (lanes 2 and 5) were probed with preimmune serum (lanes 4 to 6) or anti-35K/75K serum (lanes 1 to 3) as described in Methods. Pre-stained \( M_r \) markers (Bethesda Research Laboratories) are in lanes 1 and 4. The \( M_r \) values are indicated to the left.

Fig. 6. Specificity of affinity-purified anti-35K/75K antibodies. Nitrocellulose blots of 72 h p.i. nuclear extracts were probed with anti-35K/75K serum before (lane 1) or after preabsorption to nitrocellulose patches containing the 35K (lane 2) or 75K (lane 3) proteins as described in Methods. The control (lane 4) was probed with anti-35K/75K serum preabsorbed to nitrocellulose cut from between the 35K and 75K bands. The positions of the 35K and 75K proteins are indicated.

conditions, 5% to 10% of input label was retained on the column from comparable quantities of uninfected nuclear extracts and less than 1% of input label from infected nuclear extracts was retained on control cellulose columns lacking DNA (not shown).

The major methionine-containing polypeptides retained by the column (Fig. 4b) exhibited \( M_r \) values of approximately 110K, 93K, 83K, 75K, 35K and 18K, the smallest most probably representing host histone (compare with Fig. 2a). Coomassie Brilliant Blue staining of the same gel (Fig. 4c) revealed, in addition to putative host histones (\( M_r \) values of approx. 20K), the presence of a strongly stained band with an \( M_r \) of 35K. Chromatography of infected cell nuclear extracts on denatured DNA–cellulose columns gave similar results, although with somewhat lower overall yields (not shown).

The 75K and 35K DBPs are serologically related

Polyclonal antisera against electrophoretically purified 35K DBP were raised in rabbits as described in Methods. The antisera recognized antigens with \( M_r \) values of 35K and 75K on immunoblots of material eluted in high-salt buffer from DNA affinity columns (Fig. 5, lane 2). No immunoreactivity was detected with nuclear extracts from mock-infected cells (Fig. 5, lane 3), or when blots were probed with preimmune serum (Fig. 5, lanes 5 and 6).
To determine whether the antiserum cross-reacted with the 35K and 75K proteins because of shared antigenic determinants or due to the presence of antibodies against small amounts of 75K material contaminating the electrophoretically prepared 35K DBP, we used the affinity purification method of Smith & Fisher (1984). Nitrocellulose sheets were loaded with electrophoretically resolved 72 h p.i. nuclear extracts. Regions corresponding to the 75K and 35K bands were then cut from the sheet, incubated with antiserum under standard conditions, and the immunoglobins eluted as described in Methods. Patches cut from other regions of the sheet served as negative controls. As shown in Fig. 6, antibodies purified by preabsorption to the 35K band recognized both the 35K and 75K proteins on subsequent immunoblots (Fig. 6, lane 2). Similarly, both species were recognized by antibodies preabsorbed to the 75K band (Fig. 6, lane 3). The 35K and 75K DBPs can therefore be considered to have related epitopes.

The kinetics of appearance of the 75K and 35K proteins in nuclear and cytoplasmic extracts of CTHV-infected cells as detected by immunoblotting is shown in Fig. 7. The 75K protein was found only in nuclear extracts in which it was detectable by 24 h p.i. The 35K protein, on the other hand, appeared in both nuclear and cytoplasmic extracts at 48 h p.i. The earlier appearance of the 75K species compares favourably with the data obtained for methionine-containing polypeptides (Fig. 2b).

Virion proteins of CTHV and herpesvirus sylvilagus

The 35K antigen was detected in preparations of sucrose gradient-purified CTHV virions (Fig. 8). For comparative purposes we also examined herpesvirus sylvilagus virions which had been grown on the same cells and purified by the same procedure (Methods). As seen in the amido black-stained electrophoretic profiles in Fig. 8(a) the protein patterns of the virion preparations (lanes 1 and 4) showed substantial similarities, particularly with respect to some prominent species in the \( M_r \) range 43K to 100K. There were differences however, the most
Fig. 8. Comparison of CTHV and herpesvirus sylvilagus virion proteins by staining and immunoblotting. Sucrose gradient-purified virions were incubated with NP40 and separated into supernatant and pellet fractions as described in Methods. Electrophoretically separated polypeptides were transferred to nitrocellulose and stained with amido black (a). The nitrocellulose sheet was then probed with anti-35K/75K serum (b). The latter treatment caused the sheet to shrink about 10% in both dimensions. Lanes 1 to 3, CTHV; lanes 4 to 6, herpesvirus sylvilagus. Lanes 1 and 4, intact virions; lanes 2 and 5, NP40 supernatants; lanes 3 and 6, NP40 pellets. Lanes M, Mr markers. The asterisk to the left of CTHV lane 1 in (a) indicates the position of the immunoreactive 35K band shown in (b). Mr values are indicated to the left.

obvious of which were to be found in the regions comprising 100K to 150K and 30K to 40K. Notably, the major capsid proteins near 150K did not comigrate; the CTHV protein migrated slightly ahead of its presumed counterpart in herpesvirus sylvilagus. With a few possible exceptions, the stained herpesvirus sylvilagus virion proteins shown here corresponded reasonably well to the [35S]methionine-labelled virion proteins shown in Fig. 6 of Cohrs & Rouhandeh (1982).

In order to determine the location of the 35K protein in the CTHV virion, preparations were treated with the non-ionic detergent NP40 and centrifuged to separate capsid/tegument structures from soluble envelope proteins; the same procedure was applied to herpesvirus sylvilagus virions, again for comparative purposes. For CTHV, the majority of the virion proteins partitioned with the capsid/tegument fraction (Fig. 8a, lane 3), although two prominent polypeptides of approximately 60K and 75K were exclusively associated with the envelope fraction (Fig. 8a, lane 2). The results were less clear for herpesvirus sylvilagus, perhaps due to partial degradation of capsid/tegument structures, but two proteins of similar Mr also appeared to be restricted to the soluble fraction. When the stained nitrocellulose sheet of Fig. 8(a) was probed with anti-35K/75K serum, the 35K protein was detected only in virions and in the capsid/tegument fraction of CTHV (Fig. 8b, lanes 1 and 3). No cross-reaction was observed with herpesvirus sylvilagus virion proteins (Fig. 8b) or with extracts of herpesvirus sylvilagus-infected cells (data not shown).

Virus-specific phosphoproteins

Nuclear extracts of infected cells labelled with [32P]orthophosphate late in the infectious cycle contained six phosphorylated polypeptides which were not detectable in mock-infected cells (Fig. 9a). Four of these comigrated with the 93K, 83K, 75K and 35K species previously identified as virus-induced, methionine-containing polypeptides. One of the remaining two,
Fig. 9. Comparison of $^{35}$S-labelled (lanes 3 and 4), $^{32}$P-labelled (lanes 1 and 2) and antigenic polypeptides in nuclear extracts of CTHV-infected cells. Labelling, as described in Methods, was from 50 h to 72 h p.i. (a) Direct autoradiography of gel. (b) The same extract as in (a), but an independent gel was transferred to nitrocellulose and autoradiographed. (c) Nitrocellulose blot in (b) probed with anti-35K/75K serum. Lanes 1 and 3, infected cell extracts; lanes 2 and 4, mock-infected cell extracts. Mr values are indicated.

with an $M_r$ of approx. 60K, comigrated with a methionine-containing polypeptide found in both infected and mock-infected nuclei, and the second, with an $M_r$ of approx. 32K, did not have a readily detectable methionine-containing homologue.

In order to examine more closely the relationship between the radioactively labelled 35K and 75K polypeptides and the 35K and 75K antigens detected on immunoblots, the appropriately labelled nuclear extracts were electrophoresed and transferred to a nitrocellulose sheet. The sheet was first probed with the anti-35K/75K serum, photographed (Fig. 9c), and then autoradiographed (Fig. 9b). Superposition of the autoradiogram and the immunoblot provided a perfect coincidence of the relevant radioactive and immunostained bands.

DISCUSSION

This study has led to the detection of several CTHV-induced polypeptides in infected cells. A total of six methionine-containing species with $M_r$ values of approximately 150K, 110K, 93K, 83K, 75K and 35K could be unambiguously identified, all of which were found because they accumulated in the nucleus late in the infectious cycle (Fig. 2). However, due to a seemingly unabated rate of host protein synthesis throughout infection, it has proved difficult to recognize virus-specific polypeptides in the cytoplasm. Two apparent exceptions are the 150K and 35K polypeptides (Fig. 2a), although it can not be excluded that their presence may simply be due to leakage from nuclei during the subcellular fractionation procedure. A relatively slow and
inefficient shut-down of host protein synthesis is also observed following infection by herpesvirus sylvilagus (Patick & Hinze, 1984) and herpesvirus saimiri (Modrow & Wolf, 1983; Randall et al., 1983), the disadvantages of which in the latter case have been partly overcome by the use of phorbol esters (Modrow & Wolf, 1983). Since our major interest has been a description of the nuclear DBPs we have not tried this approach, although a more complete investigation of CTHV-specific protein synthesis may well require such a procedure.

Five of the six CTHV-specific methionine-containing polypeptides, the 110K, 93K, 83K, 75K and 35K species, are also DBPs as judged by their retention on DNA-cellulose columns (Fig. 4). Note, however, that the attribution of DNA-binding properties is a matter of convention, since we have not purified the individual polypeptides in order to determine their intrinsic binding properties. It may turn out that one or another of these proteins is retained indirectly through interaction with a bona fide DBP, a situation for which there are precedents in other virus–host systems (Freeman & Powell, 1982; Vaughan et al., 1984; Ko et al., 1986).

In terms of relative protein concentration as measured by staining (rather than by isotope incorporation) we have consistently found the 35K species to be the most abundant of the DBPs. A very rough estimate can be made concerning the quantity of 35K DBP in the nuclei 72 h p.i. Typical yields of total protein in high-salt nuclear extracts have been in the region of 30 to 40 μg per 10⁶ nuclei. From numerous experiments the recovery of input protein from the DNA-cellulose column was at least 10%, and visual inspection of Coomassie Brilliant Blue-stained gels (e.g. Fig. 4c) suggests that the 35K protein could represent as much as 10% of the eluted material, or 300 to 400 ng per 10⁶ nuclei. This corresponds to 5 × 10⁶ to 7 × 10⁶ molecules per nucleus, or approximately 10% of the abundance of cellular core histones (50 × 10⁶ to 60 × 10⁶ copies of each histone per nucleus), and indicates that the 35K DBP is a major structural protein of infected cell chromatin.

The 35K and 75K DBPs clearly share related antigenic determinants (Fig. 6). Since nuclear accumulation of the 75K DBP precedes that of the 35K DBP (Fig. 7), the most straightforward interpretation of this relatedness is that the 35K protein is derived from the 75K protein by, for example, proteolytic modification or alternative modes of expression of a single gene. However, as yet we have no information with respect to the nature of the antigenic determinants responsible for the cross-reactivity, and hence to the extent to which the overall amino acid sequences of these proteins are related. Instances have been reported in which polyclonal antisera directed against a purified protein cross-reacted with restricted, presumably immunodominant domains in divergent proteins, for example the nucleotide-binding sites of dehydrogenases (Katiyar & Porter, 1983) or the tetrahydrofolate-binding regions of eukaryotic and prokaryotic tetrahydrofolate syntheses (Staben & Rabinowitz, 1983). An additional example, detected with a polyclonal anti-nonapeptide serum, is that of the cross-reactivity of limited epitopes within the otherwise non-homologous subunits of the herpes simplex virus ribonucleotide reductase (Cohen et al., 1986). Preliminary proteolytic fingerprinting data (C. Cajeau-Feroldi, unpublished data), however, indicate that the relatedness of the 35K and 75K proteins may indeed extend to the level of primary structure.

Both the 35K and the 75K DBPs comigrate electrophoretically with prominent virus-specific phosphoproteins (Fig. 9). On this basis we provisionally propose that these as well as two additional virus-specific nuclear proteins, the 93K and 83K DBPs which also comigrate with phosphorylated polypeptides (Fig. 9), are phosphoproteins. In the case of the 35K/75K DBPs, a direct test, such as specific immunoprecipitation of the relevant phosphorylated species from crude extracts, has unfortunately not been possible due to the lack of precipitating activity of the anti-35K/75K sera (not shown). Only two of the virus-specific methionine-containing peptides detected are apparently not phosphorylated in vivo; the 150K protein, which is possibly the major capsid protein (Fig. 8a), and the 110K DBP.

The virus described here was named CTHV in order to signify its origin as being independent of the other known cottontail rabbit herpesvirus, herpesvirus sylvilagus (Hinze, 1971). Whether this formality will continue to be warranted as new comparative data are gathered remains to be seen. The comparison of virion polypeptides shown in Fig. 8 (a) demonstrates that the two viruses are similar but not identical, and we have been unable to detect a herpesvirus sylvilagus
protein in the virion (Fig. 8b) or in infected cells (unpublished data) that cross-reacts with our anti-35K/75K antisera. Although not revealing the precise extent of differences between CTHV and herpesvirus sylvilagus, the present results, as well as differences in restriction enzyme fragment patterns of the two viral genomes (Medveczky et al., 1984; Rouhandeh & Cohrs, 1987; J. Cebrian et al., personal communication), argue against the possibility that they are simply independent isolates of the same strain of virus. The ultimate conclusion as to the relatedness of CTHV, herpesvirus sylvilagus and herpesvirus saimiri will require nucleotide sequence comparisons, and we are currently attempting to isolate and characterize the CTHV genes corresponding to the DBPs described here.

This article is dedicated to the memory of Peter Wildy. The work described here was supported by grants from the Centre National de la Recherche Scientifique (ATP 'Organisation et Expression du Genome dans les Cellules Eucaryotes') and the Association pour la Recherche sur le Cancer at Villejuif.

REFERENCES


CTHV DNA-binding proteins


(Received 4 January 1988)