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The terminal protein of minute virus of mice is an 83 kilodalton polypeptide linked to specific forms of double-stranded and single-stranded viral DNA

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A new assay (label transfer from DNA to protein) enabled the identification of a terminal protein (TP) in nucleoprotein complexes extracted from cells infected with the parvovirus, minute virus of mice, MVM. In SDS-PAGE, TP migrates as a major band at 83 kDa, with a minor 65 kDa component, each of which exactly co-migrates with the cellular forms of the virally coded polypeptide NS-1. In parallel, the analysis of nucleoproteins by SDS-agarose gel electrophoresis allowed us to observe that the major species of viral DNA molecules (mRF, dRF and ssDNA) are all present in the form of DNA-protein complexes. Three forms of mRF DNA were identified, two of which are protein-associated and one which appears to be protein-free.

Parvovirus; Nucleoprotein; Terminal protein; Label transfer

1. INTRODUCTION

A terminal protein (TP) has been reported to be covalently linked to monomeric duplex DNA of three rodent parvoviruses, namely H-1, MVM and Kilham rat virus (KRV) [1–4]. These viruses have a number of properties in common [5]. Their genomes are linear, single-stranded DNA molecules, about 5000 nucleotides long, with palindromic sequences at each end, capable of folding into hairpins, which probably play an important role in viral DNA replication. The intracellular viral DNA comprises linear duplexes of single and double-unit lengths and progeny single-stranded DNA. The duplex palindromic termini exist in two conformations: they either show a hairpin, turn-around conformation or an extended conformation corresponding to an open-ended duplex.

Astell et al. [2] have shown that the extended form of MVM RF frequently bears a protein at its 5′ end. Until recently, nothing was known about its origin. Previously published sizes of TPs of parvovirus H-1 and MVM were 60 kDa [1,2,4]. For KRV, two proteins of 90 kDa and 40 kDa have been described [3]. These differences are rather unexpected if the TPs of these three closely related viruses [5] are viral gene products. Indeed we have recently shown, in the case of MVM, that a protein of 83 kDa immunologically related to the major non-structural protein NS-1, is covalently associated with the 5′ end of most forms of intracellular viral DNA [6]. To examine MVM TPs with a procedure not based on immunological identity, we have isolated viral nucleoproteins, and characterized the TPs of MVM by developing a different experimental approach, derived from in vitro assays used for identifying topoisomerase I. This assay measures the transfer of radioactivity from ^{32}P-labeled DNA to the enzyme [7]. For these

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Abbreviations: TP, terminal protein; MVM, minute virus of mice; mRF, monomeric replicative form; dRF, dimeric replicative form; ssDNA, single-stranded DNA; RI, replicative intermediate; SDS, sodium dodecyl sulfate, PAGE, polyacrylamide gel electrophoresis

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experiments, single-stranded and double-stranded DNA-protein complexes were extensively purified from MVM-infected mouse cells labeled in vivo with [^{32}P]orthophosphate, and were then hydrolyzed with DNase I. In this way the ^{32}P label was transferred from DNA to the terminal protein. Analysis of the proteins labeled by this transfer procedure demonstrated that the terminal protein of MVM is predominantly a single 83 kDa species, entirely consistent with it being NS-1, and providing further independent evidence against the existence of the major 60 kDa form previously reported [4]. Furthermore, analysis of the electrophoretic properties of the nucleoprotein complexes indicates that most of each of the intracellular viral DNA species identified as mRF, dRF and ssDNA, exist as DNA-protein complexes in infected cells.

2. MATERIALS AND METHODS

2.1. *Purification of viral DNA-protein complexes*

Mouse A9 cells were infected with MVMp, the prototype strain of MVM, at a high multiplicity of infection and labeled for 7 h between 13 and 20 h after infection, in culture medium devoid of unlabeled phosphate and containing [^{32}p]orthophosphate (0.1 mCi/ml). Cells were lyzed by incubation at 37 °C for 1 h in a lysis buffer, modified from Shoyab and Sen [9], containing 1% SDS, 6 M urea and 1 mM phenylmethylsulfonyl fluoride. Cellular DNA was then pelleted by ultracentrifugation and the supernatant recovered. Purification was achieved by a three-step procedure: gel filtration in SDS on a Sepharose CL-4B column according to Bodnar et al. [10], isopycnic separation in a density CsCl gradient containing 4 M guanidine hydrochloride according to Robinson et al. [11] and sedimentation in a preformed 5–20% sucrose gradient containing 4 M guanidine hydrochloride according to Lavelle and Li [12].

2.2. *SDS-agarose gel electrophoresis*

After chromatography, isopycnic separation and velocity sedimentation, DNA-protein complexes were analyzed on an 0.8% agarose slab gel containing 0.2% SDS [13]. Before loading, samples were heated at 80 °C for 10 min in normal sample buffer containing 2% SDS and 0.15 M LiCl. DNA bands were revealed either by staining with ethidium bromide or by autoradiography with Kodak XAR-5 film.

2.3. *Protein gel electrophoresis*

10^5 cpm (about 3 μg) of purified ^{32}P-labeled DNA-protein complex were hydrolyzed with 100 μg/ml of RNase-free-DNase I (Bethesda Research Laboratories, Gaithersburg, MD, USA) in 50 mM sodium acetate pH 6.5, 10 mM $MgCl_2$, 2 mM $CaCl_2$, for at least 2 h at 37°C. Protein samples were then analyzed in discontinuous SDS-PAGE according to Laemmli [14] using a 7.5% acrylamide resolving gel. Radioactivity was detected by autoradiography.

3. RESULTS

3.1. *Diversity among MVM nucleoprotein complexes*

Viral DNA-protein complexes present in infected cells were labeled in their DNA component with [^{32}P]orthophosphate and then purified in a three-step procedure, during which they were analyzed by SDS-agarose gel electrophoresis. Fig.1a shows the results obtained with fractions collected from the CsCl gradient. All viral DNA species (mRF, dRF and ssDNA) were recovered in the gradient peak (lanes 3–9). Three discrete bands, migrating close to the position of mRF DNA are distinguishable in lane 1. These are most easily seen

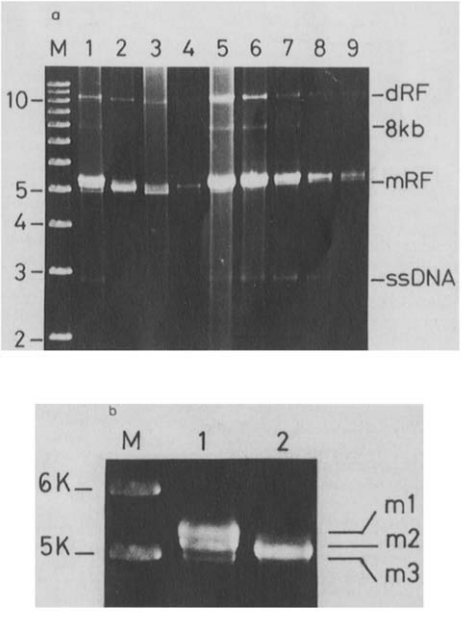

Fig.1. (A) SDS-agarose gel showing MVM nucleoprotein complexes collected from a guanidine hydrochloride-CsCl gradient and visualized by ethidium bromide staining. Lanes 3–9 are aliquots of radioactive fractions of the gradient peak from the densest (lane 3) to the lightest (lane 9) CsCl concentration. A mixture of fractions 3, 6 and 7 was electrophoresed either before (lane 1), or after (lane 2) proteinase K hydrolysis (1 mg/ml, 1 h at 37 °C). Lane M, double-stranded DNA markers (sizes in kilobases are indicated on the left). 8 kb, the 8 kb form ([15]; see text). (B) Enlargement of the mRF region of lanes m1 and 2 from panel A. m1, m2, m3 refer to the 3 DNA bands visible in lane 1.

in fig.1b, which shows an enlargement of the mRF region in which these three bands migrate. Each component of this triplet was differently distributed across the gradient peak: fractions corresponding to the highest densities contained the fastest migrating components (fig.1a, lanes 3 and 4), while components with lower electrophoretic mobilities appeared in fractions of decreasing densities (lanes 5–9). After deproteinization these 3 bands were replaced by a single species, comigrating with mRF DNA (lane 2). These results suggest that among the three molecular species, the fastest migrating component is free mRF DNA and that the two additional bands migrating more slowly are nucleoproteins containing mRF and differing in their protein content. Comparison of lanes 1 and 2 shows that ssDNA and dRF also produce differences in their electrophoretic profiles when the samples were (lane 2), or were not (lane 1), deproteinized. The additional band migrating more slowly than dRF (lane 1) disappeared after deproteinization (lane 2). Deproteinized ssDNA (lane 2) migrated faster than the corresponding ss nucleoprotein (lane 1).

Fractions collected from a guanidine hydrochloride-sucrose gradient were also analyzed by SDS-agarose gel electrophoresis (fig. 2). In such a gradient ssDNA sediments faster (lanes 1–8) than mRF (lanes 16–20). Intermediate fractions (lanes 9–13) contain a mixture of dRF, mRF, RI and 8 kb form. This latter possibly corresponds to that characterized by Faust and Gloor [15] as comprising dimer-length molecules which contain long stretches of ssDNA. Here again deproteinization of selected samples increased the electrophoretic mobility of their predominant components, for ex-

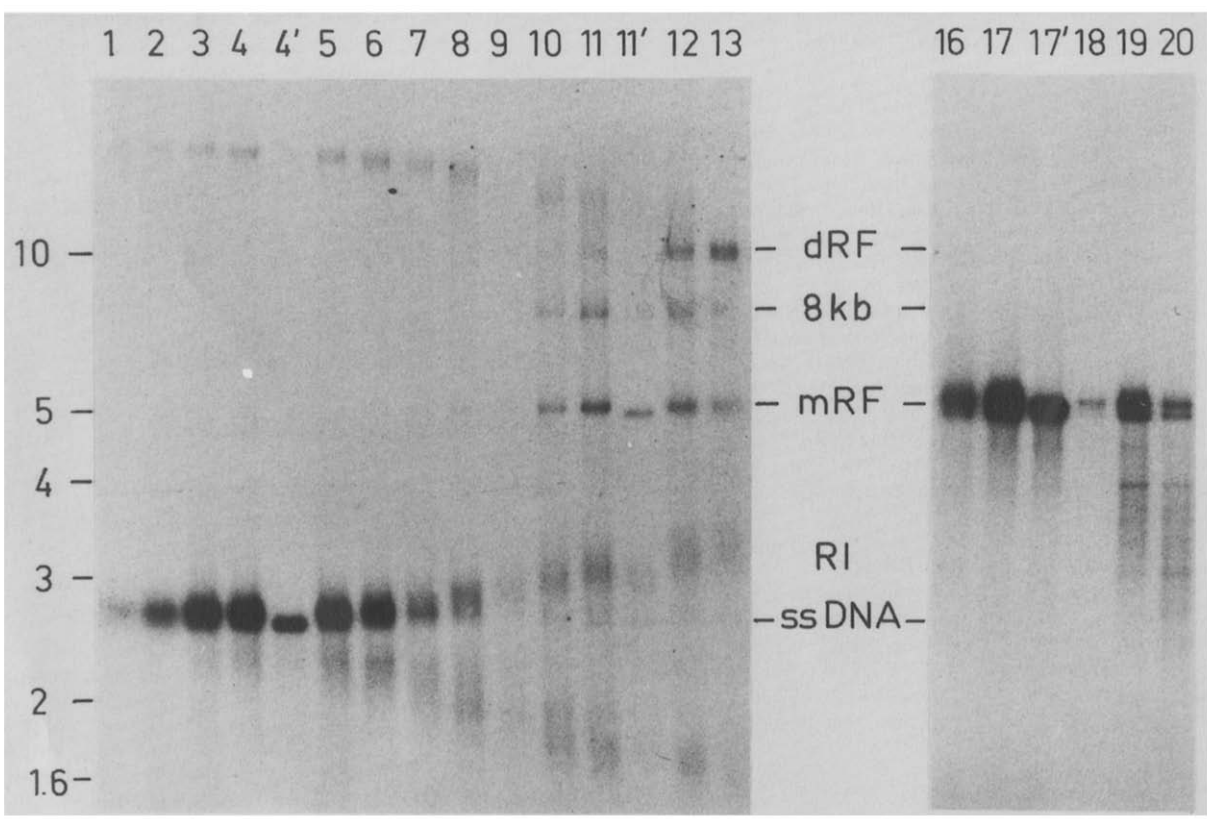

Fig.2. Autoradiograph of an 0.8% agarose-SDS gel showing labeled DNA in fractions from a guanidine hydrochloride:sucrose gradient. Fractions are numbered from the bottom (lane 1) to the top (lane 20) of the gradient. Samples from selected fractions (lane 4′, 11′ and 17′) were hydrolyzed with proteinase K (1 mg/ml, 1 h at 37 °C) before loading. Sizes (in kb) of double-stranded DNA markers, run in a parallel lane, are given on the left.

ample, ssDNA in lane 4′ and mRF in lanes 11′ and 17′. From the results shown in figs.1 and 2 it appears that a major component of each intracellular MVM DNA species (mRF, dRF and ssDNA) is associated with protein.

3.2. *Analysis of proteins present in DNA-protein complexes*

To obtain information about the molecular mass(es) of the polypeptide component(s) of these DNA-protein complexes, ^{32}P-labeled nucleoprotein samples were extensively hydrolyzed with DNase I, then analyzed in protein gels. The autoradiogram shown in fig.3 corresponds to a gel loaded with DNA-containing samples collected from a CsCl-guanidine hydrochloride density gradient, such as that shown in fig.1. Two bands revealed by their $^{32}PO_4$ label can be seen. The major one, which migrates at 83 kDa, was obtained routinely. An additional, much fainter, band at around 65 kDa, was also occasionally observed (lane 1). The detection of both of these bands in the protein gel was absolutely dependent upon prior DNase I degradation (data not shown). That these two bands correspond to protein molecules was demonstrated by their degradation by proteinase K (lane 2). Pools of single-stranded (fractions 1–8) and mRF (fractions 16–20) nucleoproteins separately collected from a sucrose gradient similar to the one shown in fig.2 were also analyzed for their protein content, and, once again, the predominant labeled polypeptide observed in both samples was 83 kDa (data not shown). Significantly these two polypeptide species mirror exactly, in both molecular mass and relative abundance in the infected cell, the full-length, 83 kDa, major viral non-structural protein NS-1, and its stable, in vivo cleavage product, which lacks approximately 19 kDa from the C-terminus of the 83 kDa form [5,6,8].

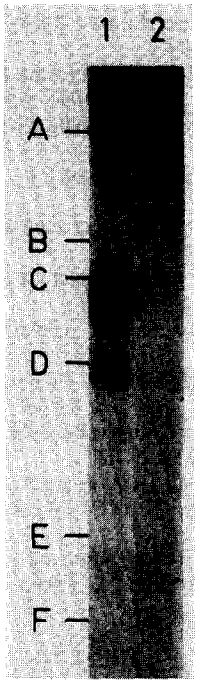

Fig.3. Autoradiograph of an SDS-PAGE analysis of ^{32}P-labeled nucleoprotein complexes isolated from guanidine hydrochloride-CsCl gradient and hydrolyzed with: lane 1, DNase I; lane 2, DNase I then proteinase K. Sizes of protein standards run in a parallel lane are: (A) 200 kDa; (B) 116.25 kDa; (C) 92.5 kDa; (D) 66.2 kDa; (E) 45 kDa; (F) at 31 kDa, is the DNase I used to hydrolyze the sample.

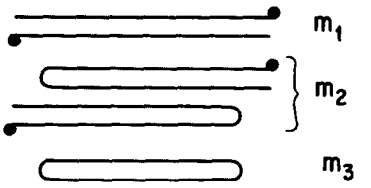

Fig.4. Suggested structures for the components of the three bands migrating in the position of mRF, as shown in fig.1b. Although four structures are depicted, two of them, designated m_2, would be expected to co-migrate in an SDS-agarose gel. ●: TP.

4. DISCUSSION

MVM nucleoproteins were labeled in vivo with [^{32}P]orthophosphate, purified and hydrolyzed with DNase I, with the aim of isolating the terminal protein. Subsequent analysis in SDS-PAGE revealed one major protein band of 83 kDa. It is important to point out that DNA-protein complexes were extracted and purified without any enzymatic proteolysis and in the presence of protease inhibitor and several kinds of denaturing agents (SDS, urea, guanidine). Purification based on several properties of macromolecules was achieved according to the three-step procedure described above. This allowed us to demonstrate that the terminal protein is predominantly an 83 kDa protein, and to show that not only mRF but also dRF and ssDNA were

extracted as DNA-protein complexes from MVM-infected cells. Interestingly, three mRF subspecies which differ in their protein content were observed by SDS-agarose gel electrophoresis. The extensive purification used strongly suggests that the viral nucleoproteins are composed predominantly of DNA and TP. We therefore propose the following structures for mRF subspecies (fig. 4): m_3, comprising free DNA without TP would show a configuration with two hairpin termini, while the m_2 species would have one TP molecule and one extended terminus, and m_1 would have two TP molecules and two extended termini. In this analysis, dRF behaves as two subspecies in SDS-agarose gel electrophoresis, the slower migrating one being linked to TP. A fraction of ssDNA was also found linked to TP. Recently we have confirmed these observations, and characterized, by immunological means, the TP present in these structures as the major viral non-structural protein NS-1 (Cotmore, S.F., Gunther, M. and Tattersall, P., submitted to J. Virol).

The 83 kDa protein, visualized in SDS-PAGE, was detected in preparations containing all viral DNA species as well as in single-stranded or mRF nucleoproteins separately recovered from the sucrose gradient. The conditions under which the protein was labeled and isolated strongly suggest a stable association between the 83 kDa protein and DNA, most probably a covalent link. The protein labeling with ^{32}P is of particular interest, as it arose from the phosphate remaining attached to the protein after DNA hydrolysis. However, we cannot exclude a contribution to the label due to phosphorylation of the protein in vivo. That the 83 kDa protein could correspond to another protein than TP is highly improbable. Indeed such a putative protein would have been labeled with ^{32}P, tightly associated to DNA and present in relatively high proportions in all our preparations of nucleoproteins.

In conclusion, we have isolated a phosphorylated, DNA-linked, 83 kDa protein which is very likely the terminal protein previously identified as the major viral non-structural protein NS-1, and have shown that this protein is the major polypeptide species presumably covalently associated with intracellular MVM DNA.

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