Expression and DNA binding of budgerigar fledgling disease virus large T antigen

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Budgerigar fledgling disease virus (BFDV) represents the first non-mammalian member of the polyomavirus genus and possesses uncommon structural and biological properties. Recombinant baculoviruses were constructed to express BFDV small t antigen, large T antigens, as well as a large T deletion mutant T~ and β-galactosidase-T~ fusion proteins to high levels in infected insect cells. A recombinant virus containing a genomic copy of the BFDV early region was used for small t antigen expression, and corresponding intron-deleted cDNAs for production of large T antigen derivatives. Recombinant T as well as authentic T antigen proteins from infected chicken embryo fibroblasts were purified using both immunoaffinity and DNA affinity column chromatography. We present evidence that the large T antigen interacts specifically with DNA sequences present in the non-coding region of BFDV; by indirect DNA immunoprecipitation mapping and DNase I footprinting, four regions including 12 DNA-binding sites have been determined that cover most of the BFDV non-coding region. The T antigen binding pattern observed suggests a protein–DNA interaction system considerably different from those of simian virus 40 and other polyomaviruses.

Introduction

Budgerigar fledgling disease virus (BFDV) represents the first avian isolate of a polyomavirus (Lehn & Müller, 1986; Müller & Nitschke, 1986; Rott et al., 1988). It is closely related to the two well characterized papovaviruses, simian virus 40 (SV40) and murine polyomavirus (Py) as well as to other viruses in this group, with which it shares extensive structural as well as functional similarities (Rott et al., 1988). In contrast to mammalian polyomaviruses, BFDV is associated with an acute disease resulting in the death of affected birds (Davis et al., 1981); on the other hand, BFDV is also able to transform primary embryo hamster fibroblast cells in vitro (Lehn & Müller, 1986).

The genome of BFDV has recently been sequenced and characterized (Rott et al., 1988; Stoll et al., 1993). Similar to other polyomaviruses, the 4981 bp DNA genome can be divided into an early region and a late region. The deduced amino acid sequences from the early region, which encodes the large T and small t antigens, revealed some remarkable differences from other polyomaviruses, including a considerable reduction in size of the large T antigen. The late region contains a standard pattern of the three genes coding for the structural proteins VP1, VP2 and VP3, as well as open reading frames coding for proteins with largely unknown functions, agnoproteins. Finally, the replication origin structures and transcriptional control elements in the non-coding region of BFDV show fundamental deviations from those of all other polyomaviruses (Rott et al., 1988).

It is well known that SV40 large T antigen binds to specific sequences in the viral control region and regulates early and late SV40 transcription; it also plays an essential role in the initiation of viral DNA replication (for review see Pipas, 1992). Unlike SV40, replication from the origin of the Py genome requires the presence of an enhancer element (de Villiers et al., 1984; Prives et al., 1987). Although the functions of the BFDV large T antigen are presently not known in detail, analogy with polyomavirus suggests that it will act as a major regulatory protein governing the BFDV viral infection cycle. It has been speculated that it could be involved in the regulation of initiation of viral DNA replication as well as viral RNA transcription (Rott et al., 1988).

Recently, a helper-independent baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV) expression system has been developed and adapted to the overproduction of a variety of eukaryotic proteins in insect cells (Smith et al., 1983; Luckow & Summers, 1988). In this system, the foreign gene is inserted in the
place of the AcNPV polyhedrin gene, which is non-

essential for virus production in cell culture. Expression of the cloned gene is under the control of the strong and late polyhedrin gene promoter. This expression system has been used for the construction of four recombinant baculoviruses that produce BFDV small T antigen, large T antigen, a deletion mutant of T antigen (T\(_d\)) and a \(\beta\)-galactosidase-T\(_d\) fusion protein. Recombinant BFDV T antigen purified using both immunoaffinity and biotin–oligonucleotide–streptavidin affinity columns was found to bind specifically to the BFDV non-coding region sequence. By indirect DNA immunoprecipitation mapping (McKay, 1981) and DNase I footprinting (Galas & Schmitz, 1978) four regions including 12 DNA-binding sites have been determined that cover most of the BFDV non-coding region; their location suggests a protein–DNA interaction system different from those of SV40 and other polyomaviruses.

**Methods**

**Cells and viruses.** A permanent cell line (S9) derived from *Spodoptera frugiperda* was propagated in TC-100 medium (Gibco BRL) supplemented with 10% fetal calf serum at 27 °C. Wild-type AcNPV was amplified by infection of S9 cells. The procedures outlined by Summers & Smith (1987) were used for virus propagation and purification. Chicken embryo fibroblast (CEF) cells were used for preparation of BFDV as previously described (Lehn & Müller, 1986; Müller & Nitschke, 1986). Cells plated onto a 90 mm dish were infected with 

**Plasmids.** The baculovirus transfer vector pAc611 (Doerfler, 1986; Luckow & Summers, 1988) with the polyhedrin promoter and multiple cloning sites was used to construct recombinant transfer plasmids. Four recombinant transfer plasmids were constructed as shown in Fig. 1. In the case of pAc-FL644, an XbaI–EcoRI DNA fragment (2 kb) containing a genomic copy of the BFDV early region was cloned into the XbaI–EcoRI sites of the baculovirus transfer vector. Plasmid pAc-FL873 was constructed using a cDNA copy (1.8 kb) of the BFDV large T antigen gene in which an intron with 195 nucleotides (nt) ranging from nt 4733 to 4537 had been removed. Plasmid pAc-HL643 was designed to become deleted from the BFDV early region in accordance with initial predictions about the intron boundaries in the large T antigen (Rott et al., 1988). For the construction of pAc-FL668 a BamHI–EcoRI DNA fragment (4.9 kb) containing a hybrid gene encoding the \(\beta\)-galactosidase-T\(_d\) fusion protein was isolated from pH723 (Stoll et al., 1993) and then inserted into the baculovirus transfer vector.

For the DNA binding assay, a 770 bp SfiI–PstI fragment (positions 4957 to 747), containing the putative BFDV replication origin and transcriptional control elements (Rott et al., 1988) was cloned into the polylinker of cloning vector pUC19 (New England Biolabs) to create plasmid pH714. For DNA binding (gel retardation and DNase I footprint assays), a smaller 230 bp SfiI–HindIII fragment (positions 4957 to 203) containing the four BFDV large T antigen binding sites was first filled in to create a HindIII blunt-ended site, and then cloned into SstI–Smal of pUC19 to generate plasmid pH780.

**Production and selection of recombinant baculoviruses.** Recombinant baculoviruses were prepared in two steps: by construction of recombinant transfer plasmids in *E. coli*, followed by homologous recombination in vivo between wild-type AcNPV DNA and hybrid transfer plasmid DNAs. S9 cells were cotransfected with 1 \(\mu\)g of wild-type AcNPV DNA and 2 \(\mu\)g of specific transfer plasmid DNA by the cationic liposome-mediated transfection method (Lipofectin reagent; Gibco BRL). Eighty percent confluent cells were washed twice with PBS and once with Opti-MEM medium (Gibco BRL). Lipofectin and DNA were separately diluted into Opti-MEM. For each 60 mm dish, 2 to 10 \(\mu\)g of combined DNAs were added to 1-5 ml of medium, and 30 \(\mu\)g of Lipofectin was added to a second 1-5 ml volume of medium. These two solutions were mixed and applied onto the cells. After 4 h incubation at 27 °C the transfection mixture was removed and 5 ml of fresh TC100 medium supplemented with 10% serum was added. Progeny viruses were harvested from transfected cultures after 5 days incubation at 27 °C, and were plated on S9 cells. Plaques generated by recombinant viruses were purified in three rounds of plaque hybridizations with a \(^{32}\)P-labeled specific BFDV DNA probe and in addition could be identified by visual screening for an occlusion-negative phenotype. Recombinant viral DNAs were isolated and then verified in the pertinent region by restriction enzyme analysis. Therefore, recombinant viruses derived in this way from pAc-FL873, pAc-HL643, pAc-HL644 and pAc-HL668 are referred to as Ac-FL873, Ac-HL643, Ac-HL644 and Ac-HL668, respectively.

**Analysis of recombinant proteins by SDS–PAGE and immunoblotting.** S9 cells were infected at a high multiplicity (m.o.i. of about 5 p.f.u./cell) with wild-type AcNPV or with recombinant viruses. Virus-infected cells in a 90 mm dish were collected by centrifugation, washed twice with PBS and lysed in 0.5 ml of lysing buffer (10 mM-Tris–HCl pH 7.5, 50 mM-NaCl, 5 mM-MgCl\(_2\), 0.5% NP40, 0.1 mM-PMSF). After centrifugation the pellet was resuspended in 0.5 ml of RIPA buffer (10 mM-Tris–HCl pH 7.5, 150 mM-NaCl, 1% SDS, 1% NP40, 1% deoxycholate, 0.1 mM-PMSF) for final extraction. For the analysis of proteins the solubilized cell lysates and RIPA supernatant fluids were mixed with an equal volume of double-strength concentrated Laemmli sample buffer (Laemmli, 1970). Samples were boiled for 5 min and submitted to electrophoresis on SDS–polyacrylamide gels. For immunoblot analysis, proteins were transferred electrophoretically from the polyacrylamide gels onto Immobilon PVDF membranes (Millipore) after SDS–PAGE. The membranes were soaked in blocking buffer (5% skim milk in PBS) at room temperature for 2 h and were then reacted with anti-T polyclonal antiserum (Stoll et al., 1993) diluted 3500-fold in blocking buffer at 4 °C overnight. After washing three times with washing buffer (0.05% SDS, 0.1% Triton X-100, 0.5% BSA in PBS) antigenic proteins were visualized using horseradish peroxidase-conjugated anti-rabbit IgG (Amersham) and 3,3'-diaminobenzidine (0.3 mg/ml) as a substrate after incubation at room temperature for 5 min.

**Subcellular fractionation.** Subcellular fractions of infected S9 cells were prepared essentially as described (Grossman et al., 1989). The cells were harvested 2-5 days post-infection (p.i.) and washed once with ice-cold PBS. All further steps were performed on ice or at 4 °C. To obtain cytoplasmatic fractions, the cells were suspended in isotonic STM buffer (250 mM-sucrose, 10 mM-Tris–HCl pH 8.0, 10 mM-MgCl\(_2\), 0.1 mM-PMSF) for 10 min, disrupted in a Dounce homogenizer with 20 strokes, and supernatants were collected after centrifugation at 13000 r.p.m. for 30 min. For the preparation of non-nuclear membrane fractions, the cells were disrupted in hypotonic buffer H (10 mM-Tris–HCl pH 8.0, 1 mM-DTT, 1 mM-EDTA, 0.1 mM-PMSF) by 20 strokes in a Dounce homogenizer. After low-speed centrifugation
Fig. 1. Structure of recombinant pAc-transfer plasmids. pAc-HL873 was constructed using a cDNA copy of the BFDV large T antigen gene. In pAc-HL643, a 330 bp fragment was deleted from the BFDV early region. pAc-HL644 contains a complete BFDV early region. pAc-HL668 contains a hybrid gene encoding a β-galactosidase–T₄ fusion protein. An arrow indicates the direction of transcription from the polyhedrin promoter. PA, polyadenylation sites; aa, amino acids.

Protein purification by immunoaffinity column chromatography. The protocol for preparation of an antibody-Sepharose column was provided with an ImmunoPure IgG Orientation Kit (Pierce), which was used with modifications. Briefly, 2 ml of ImmunoPure immobilized Protein A beads were equilibrated with 5 ml ImmunoPure Wash Buffer, then circulated with 5 ml anti-T polyclonal antiserum from an immunized rabbit at room temperature for 2 h. After washing twice with ImmunoPure Wash Buffer, the beads were suspended in 2 ml of ImmunoPure Cross-linking Buffer containing 13.2 mg of cross-linking agent dimethylpimeliminate dihydrochloride (DMP) and kept at room temperature for 1 h. The column was then washed once with 5 ml of ImmunoPure DMP Cross-linking Buffer, once with 2 ml ImmunoPure Blocking Buffer, twice with 10 ml of elution buffer (20 mM-triethylammonium pH 11.5, 10% glycerol), and twice with 5 ml aliquots of ImmunoPure Wash Buffer.

The cell lysate (200 μl) was mixed with 2 ml of lysis buffer containing 10% glycerol. The mixture was loaded onto the column and circulated through it at room temperature for 2 h. Thereafter the column was washed extensively with lysis buffer containing 10% glycerol before elution. The eluate was collected (in 10-0-4 ml fractions) directly into 20 μl of neutralizing buffer (1 M-KH₂PO₄/K₂HPO₄ pH 6.8, 10% glycerol).

Protein purification by biotin–oligonucleotide–streptavidin DNA
affinity column chromatography. Complementary oligodeoxynucleotides containing the major BFDV large T antigen binding sequence were synthesized in an Applied Biosystems 380B DNA synthesizer. The sequence (5'TCTTTACATAAACATGCTCATCATAAGGAATACGATATGCCATGTTTGT) was designed according to the experimental data obtained from McKay DNA binding assays (Fig. 6).

Both complementary oligodeoxynucleotides were purified from a 20% polyacrylamide gel and hybridized 1:1 to each other in 300 \( \mu l \) of buffer containing 50 mM-Tris-\( \text{HCl} \) pH 7.6, 10 mM-NaCl by incubation at 70 °C for 10 min, at 37 °C for 3 h, and at 4 °C overnight, generating duplex DNA with a 5’ protruding CTC sequence at one end, and GAG tides containing the major BFDV large T antigen binding sequence (CGGATATGCCCATGTTTGT) was designed according to the experiments.

The volume of phosphorylated at the 5’ terminus by T4 polynucleotide kinase to increase the efficiency in the following ligation reaction. The volume of the kinase reaction was adjusted to 300 \( \mu l \) by adding 30 \( \mu l \) of 10 × kinase buffer (600 mM-Tris-HCl pH 8.0, 150 mM-2-mercaptoethanol, 100 mM-MgCl\(_2\)), 30 \( \mu l \) of 10 mM-ATP, and 10 \( U \) of T4 kinase (Gibco BRL). The reaction was carried out at 37 °C for 2 h. After reaction the samples were heated to 100 °C for 5 min, then precipitated with ethanol.

The pellet was dissolved in 300 \( \mu l \) of distilled \( \text{H}_2\text{O} \) and divided into two equal portions. One portion was used for biotin-dU incorporation in a filling-in reaction at the right-hand end: 30 \( \mu l \) of 10 × labelling buffer (500 mM-Tris-\( \text{HCl} \) pH 7.5, 100 mM-MgSO\(_4\), 1 mM-DTT, 500 \( \mu g/ml \) BSA), 2.5 \( \mu l \) of 10 mM-dCTP, 15 \( \mu l \) of 1 mM-biotin-11-dUTP (Gibco BRL) and 10 \( U \) of DNA polymerase Klenow fragment (Gibco BRL) were added in 300 \( \mu l \) of reaction volume. Reaction mixtures were incubated at room temperature for 2 h, then inactivated by heating at 65 °C for 10 min. DNA duplexes were precipitated with ethanol; the pellet was washed twice with 75% ethanol to remove the non-incorporated biotin-11-dUTP and other reagents, vacuum-dried, and dissolved in 150 \( \mu l \) of distilled \( \text{H}_2\text{O} \). This biotinylated DNA fragment was equilibrated with TE buffer containing 0.2 M-NaCl and 10% glycerol, then loaded with 300 \( \mu l \) of cell lysate, circulated at room temperature for 2 h, and then washed three times with TE buffer containing 0.2 M-NaCl and 10% glycerol to remove non-binding proteins. Elution was carried out by applying TE buffer containing 2 M-NaCl and 10% glycerol. The eluate was collected in 10 0.3 ml fractions and dialysed against TE buffer containing 0.2 M-NaCl and 10% glycerol.

**DNA binding (McKay) assay.** Plasmid pH714, which carries the BFDV 770 bp SirI–Sir1 origin fragment inserted into the polylinker of pUC19, was digested with EcoRI and HindIII and end-labelled with \(^{32}P\) dCTP by using the Klenow DNA polymerase fragment. The cell lysate (100 \( \mu l \)) in lysing buffer was mixed with \(^{32}P\)-labelled, cleaved pH714 DNA in 100 \( \mu l \) of binding buffer (100 mM-Tris-\( \text{HCl} \) pH 7.4, 100 mM-KCl, 1 mM-DTT, 0.5 mM-MgCl\(_2\), 1 mM-PMSF) and incubated on ice for 30 min. Complexes were immunoprecipitated with anti-T antibody (5 \( \mu l \)) and *Staphylococcus aureus* (10 \( \mu l \)) on ice for 60 min. Samples were washed twice with NET buffer (50 mM-Tris-\( \text{HCl} \) pH 7.5, 150 mM-NaCl, 5 mM-EDTA, 0.05% NP40). Bound DNA was eluted by incubating with SDS elution buffer (10 mM-Tris-\( \text{HCl} \) pH 7.5, 1% SDS, 1 mM-EDTA) at 60 °C for 30 min. After phenol extraction, the eluate was analysed by electrophoresis on 1% agarose gels, followed by autoradiography. Various restriction enzymes were used separately to cleave the 770 bp origin fragment for the identification and localization of the large T antigen DNA-binding region. The cleaved DNA fragments were end-labelled as above for the DNA-binding immunoprecipitation assay.

**DNase I footprint.** Purified proteins were concentrated using Centricon Membrane Cones, type CF25 (Amicon), then incubated with 50 to 80 ng of end-labelled probe and 0.07 to 2.0 \( \mu g \) of poly(d\( \text{dC}\))-poly(d\( \text{dC}\)) in a volume of 20 \( \mu l \) binding buffer as above. Digestion reactions were carried out with 100 to 200 mU of DNase I (Stratagene) at room temperature for 5 min. Reactions were stopped by bringing the EDTA concentration to 10 mm. DNA was extracted, ethanol-purified, denatured at 100 °C for 3 min, and applied to 10% polyacrylamide-8 M-urea gels. Gels were then transferred to Whatman 3MM filters, dried and autoradiographed.

## Results

### Expression of recombinant proteins in Sf9 cells

To determine the synthesis of BFDV large T and small t antigens in insect cells, Sf9 cells were infected with recombinant viruses Ac-HL873 (intron removed, T antigen), Ac-HL643 (T\( \_\) deletion mutant), Ac-HL644 (intron retained, t antigen) or wild-type AcNPV. After 2-5 days of infection, the cells were harvested and lysed in lysing buffer. Following centrifugation, the pellet was extracted in RIPA buffer. Proteins were resolved by SDS-PAGE and identified by staining with Coomassie blue and immunoblotting with anti-T antisera. In contrast to both negative controls of mock-infected (Fig. 2a, lane 1) and wild-type AcNPV-infected cells (Fig. 2a, lane 6), cells infected with recombinant viruses Ac-HL873, Ac-HL643 and Ac-HL644 synthesized large T, T\( \_\) and small t antigens with approximate M\(_{r}\) values of 72K, 67K and 21K (Fig. 2a, lanes 3, 4 and 5), respectively. Since the intron segment with 195 bp, and a DNA fragment with 330 bp (Rott et al., 1988), had been removed or deleted from the BFDV early region in Ac-HL873 and Ac-HL643, respectively, no small t antigen was detectable in lysates of Ac-HL873- and Ac-HL643-infected cells. However, cells infected with Ac-HL644 which was constructed using an unspliced, genomic copy of the BFDV early region produced significant amounts of small t antigen; in this case, however, no large T antigen was observed (Fig. 2a, lane 5). These results suggest that the recombinant viruses Ac-HL873, Ac-HL643 and Ac-HL644 faithfully express the appropriate polypeptides, and that the mRNA splicing signals of the BFDV early messenger sequence are not recognized in Sf9 insect cells.

BFDV infects and propagates in CEF cells. It was of interest, therefore, to compare the amount of recombinant proteins expressed in insect cells with those of the authentic polypeptides produced in BFDV-infected CEF cells. As observed in Fig. 2(a) we estimate that the concentration of the large T and small t antigens expressed in Ac-HL873- and Ac-HL644-infected Sf9 cells is as much as two- to threefold, and 10 to 15-fold.
DNA binding of BFDV large T antigen

1271

(a)

1 2 3

67K -
43K --
30K -
20K -

(b)

Sf9 24 36 48 60 72 90
Z

T -- ~ .......... 72K
94K
67K
43K
30K
20K

Fig. 2. (a) Synthesis of BFDV large T, Td and small t antigens in recombinant baculovirus-infected insect cells. Protein samples were analysed by immunoblotting after SDS-PAGE. Lane 1, Sf9 cell lysate; lane 2, BFDV-infected CEF cell lysate; lane 3, recombinant virus Ac-HL873-infected Sf9 cell lysate; lane 4, Ac-HL643-infected Sf9 cell lysate; lane 5, recombinant virus Ac-HL644-infected Sf9 cell lysate; lane 6, wild-type AcNPV-infected Sf9 cell lysate. Positions of authentic BFDV large T antigen (TB) (74K), recombinant large T antigen (T) (72K), deletion protein Td (67K) and small t antigen (21K) are indicated. (b) Time-course of the production of recombinant large T, Td, small t and β-galactosidase–Td fusion protein in insect cells infected with recombinant viruses. Cells were collected as indicated [lanes 24 to 90 represent time (h) p.i.] and analysed by SDS-PAGE (Western blotting on 10% protein gels for large T and Td, on 15% gels for small t, and Coomassie blue staining on 7.5% gels for β-galactosidase–Td). Positions of recombinant large T antigen, deletion mutant Td, small t antigen and β-galactosidase–Td fusion protein are indicated. Lanes Sf9 and AcNPV, controls; non-transfected Sf9 cells and wild-type baculovirus-infected cells, respectively.

higher, respectively, than that observed in BFDV-infected CEF cells. Also, the recombinant baculovirus-produced large T antigen appears to be slightly smaller (72K compared to 74K) than authentic large T antigen (TB) produced in BFDV-infected CEF cells, as indicated by differences in migration on SDS-polyacrylamide gels (Fig. 2a, compare lanes 3 and 2). The differences may result from different post-translational modification patterns of the large T antigen in the two different cell types. Our preliminary observations of in vivo phosphorylation indicate that the protein expressed in insect cells is poorly phosphorylated whereas authentic protein synthesized in BFDV-infected CEF cells indeed becomes phosphorylated (H. Müller, unpublished data).

The primary purpose in constructing recombinant virus Ac-HL668 was to create a system for an easy monitoring of the level of T antigen expression because the β-galactosidase–Td fusion protein of high Mr (170K) could easily be detected on SDS-polyacrylamide gels.

To determine the optimum time of protein production and accumulation in insect cells, time-course experiments were performed. The proteins became detectable at 24 h p.i., and the maximum rate of synthesis was observed at 60 to 72 h p.i. (Fig. 2b). We estimate the yields of large T, Td and small t antigens and β-galactosidase–Td fusion protein to be around 2 to 10 mg per litre of Sf9 cells.

Protein purification

(i) Immunoaffinity column chromatography

To facilitate further studies of biological and biochemical properties of the recombinant proteins, we purified large T, Td and small t antigens expressed from recombinant baculovirus-infected insect cells, as well as large T antigen produced from BFDV-infected CEF cells. Infected cells were extracted in lysis buffer, and cell lysate was applied to columns to which specific polyclonal antibodies had been covalently linked to Protein A-Sepharose. After the unbound protein was washed away, the bound protein was eluted from the column in alkaline conditions; samples were neutralized immediately. Each column fraction was electrophoresed through the SDS-polyacrylamide gels, and the proteins were transferred to PVDF membranes for Western blotting. Fig. 3(a) shows the results of immunoaffinity purification: elution profiles of recombinant large T antigen,
Fig. 3. (a) Immunoaffinity purification of the BFDV T antigen. Fractions of purified proteins were separated on SDS-polyacrylamide gels and detected by Western blots with washing at low stringency. Lane 1, total input proteins. Lanes 2 to 10, elution fractions 2 to 10. (b) Biotin–oligonucleotide–streptavidin affinity purification. For recombinant large T antigen, lane 1 represents total input proteins, lanes 2 to 10 represent elution fractions 2 to 10. For protein T\textsubscript{a}, lane 1 represents total input proteins, lane 2, flowthrough, and lane 3, washing fraction; lanes 4 to 10 are elution fractions 2 to 8.

recombinant T\textsubscript{a} and recombinant t antigen, as well as the authentic large T antigen (T\textsubscript{B}) from BFDV-infected CEF cells.

(ii) Biotin–oligonucleotide–streptavidin affinity column chromatography

Short fragments of DNA containing a specific affinity site for a DNA-binding protein provide a powerful tool for purification. The biotin–oligonucleotide–streptavidin purification system for preparation of the column is based on the tight and essentially irreversible complex that biotin forms with streptavidin (Ausubel \textit{et al.}, 1987). The column in which biotinylated DNA oligonucleotides were linked to the streptavidin matrix was used for affinity chromatography of the BFDV T antigen. It was loaded with cell lysate, circulated, and then washed with low-salt TE buffer to remove non-binding proteins. Elution was carried out by applying high-salt TE buffer. After column purification the eluates were analysed by Western blotting (Fig. 3b). The observation that proteins T and T\textsubscript{a} were eluted in a much purified form indicates the column specificity for the large T antigen binding.

Both immunoaffinity column and biotin–oligonucleo-
Subcellular localization

It has been shown that some recombinant proteins expressed in insect cells are localized in their appropriate subcellular compartments (Miyamoto et al., 1985; Ollo & Maniatis, 1987; O'Reilly & Miller, 1988; Luckow & Summers, 1988), indicating that the specific signals of these proteins are recognized by the insect cells. To investigate the subcellular distribution of BFDV large T antigen in insect cells, Sf9 cells infected with recombinant virus Ac-HL643 were disrupted in either isotonic or hypotonic medium as described by Grossman et al. (1989). Each of the fractions obtained was analysed by SDS-PAGE and examined by Western blotting. We found that a major portion of the large T antigen was located in the nucleus (Fig. 4).

Indirect immunofluorescence experiments were carried out also to examine the subcellular localization of the large T antigen in insect cells. In contrast to the non-infected Sf9 cells and wild-type AcNPV-infected cells, specific fluorescence was predominantly found in the nucleus of Ac-HL643-infected cells (Fig. 5). Owing to high level expression of the large T antigen and the fact that the nucleus swells considerably after virus infection, it is difficult to estimate whether there is a small fraction of large T antigen remaining in the cytoplasm.
Fig. 6. For legend see opposite.
DNA-binding activity

The genome of the Papovaviridae consists of a non-coding region located between the early and late transcriptional units. The non-coding regulating region contains both the origin of replication and transcriptional control elements which interact with the large T antigen. Although the biological function of BFDV large T antigen is not yet known, comparison with SV40 or other polyomavirus T antigens suggests that it may act similarly through binding to DNA. Given other structural similarities with the SV40 T antigen this contention is supported by the observation that the BFDV large T antigen contains two stretches of potential zinc finger domains (Rott et al., 1988).

A comparison of the replication origin structures of SV40 and other polyomaviruses with the non-coding region (1 to 770 nt) of BFDV DNA revealed no evidence for a similar organization in the BFDV genome (Rott et al., 1988). This suggests that in BFDV the binding sites for large T antigen may differ from those of SV40 and other polyomaviruses. In the non-coding region of BFDV, a tandem repeated sequence has been observed; it consists of a perfectly repeated core sequence of 19 bp (5' TTACATAACATGCATTCCA 3') that can be extended up to about 45 bp by including adjacent nearly identical segments (Rott et al., 1988).

To identify and localize the DNA-binding region within the BFDV non-coding region, DNA-binding activity using the immunoprecipitation assay developed by McKay (1981) was examined, in which cell lysates were incubated with 32P-labelled DNA fragments, and T antigen–DNA complexes were precipitated by anti-T antibody. Fig. 6 (a to c) shows representative autoradiographs from these experiments and the diagram in Fig. 6(e) summarizes these results in a schematic way. As shown in Fig. 6(a), the 770 bp DNA fragment containing the putative BFDV replication origin and transcriptional control elements (Rott et al., 1988) was preferentially precipitated (lane 4). Moreover, binding of the 32P-labelled 770 bp DNA fragment was out-competed by the addition of a surplus of the unlabelled specific fragment (lanes 8 and 9), but not by addition of unlabelled pUC19 fragments (lanes 5 and 6). The results of these experiments suggest that large T antigen expressed in insect cells binds specifically to a DNA fragment containing the putative replication origin and transcriptional control elements and not to other regions of the BFDV DNA. We then used various restriction enzymes to dissect further the 770 bp DNA fragment. The initial experiments were performed using the enzyme AccI to split the 770 bp DNA fragment into two parts, resulting in a 320 bp fragment (positions 4955 to 293) and a 450 bp fragment (positions 294 to 745). The 320 bp fragment and not the 450 bp DNA fragment was found to be precipitated when lysates from Ac-HL643-infected cells were used (Fig. 6b), suggesting that the 320 bp DNA fragment contains specific binding sites. Further cleavages of this fragment in a stepwise manner from either side of its initial boundary (from the left, using Ncol or DdeI; from the right, using RsaI or HindI) led to the localization of the DNA-binding region to within the central 200 bp Ncol/HindI fragment (Fig. 6c to e).

Subsequent experiments were carried out using the enzyme NsiI which cuts twice, within the 45 bp tandemly repeated sequence (domains I and II), resulting in a complete destruction of the binding activity (Fig. 6c). This suggests that sequences around the NsiI cleavage site are involved in the binding of T antigen.
sites are important for T antigen binding. Furthermore, binding assays done with partial digestions by NsI allowed us to detect binding to single interacting sites and to determine which of the two nearly identical repeated domains might have higher, if not equal, binding activity. The results indicated that domain II (located further upstream) might be more favourable than domain I as a target for T antigen binding (data not shown).

To delineate more precisely the region of large T antigen binding, protein–DNA complexes were analysed by DNase I footprinting (Galas & Schmitz, 1978). A 32P-end-labelled 239 bp DNA fragment (Xbal–SstI from plasmid pHL870) was incubated with increasing amounts of purified protein preparations in the presence of an approximately 250-fold excess of competitor poly(dI-dC)–poly(dI-dC). The protein–DNA complex was then partially cleaved by DNase I and subjected to electrophoresis on a 10% sequencing gel. The products of Maxam-Gilbert ‘G + A’ and ‘A > C’ cleavage reactions of the same fragment were used as markers. Positions with numbers according to Rott et al. (1988) and lines for the protected regions are indicated. The right panel is a longer run of the samples analysed in the left panel. Lanes 1 to 3, controls, no protein added.

Fig. 7. DNase I footprinting. A 32P-end-labelled 239 bp (Xbal–SstI from plasmid pHL870) DNA fragment (4.3 ng) was incubated with increasing amounts (approximately 10, 25 and 40 ng) of purified recombinant large T (lanes 4 to 6), deletion protein Td (lanes 7 to 9) and BFDV authentic large Tb antigen (lanes 10 to 12) in the presence of an additional 1 μg of non-specific competitor poly(dI-dC)–poly(dI-dC). After DNase I digestion the samples were subjected to denaturing electrophoresis in a 10% polyacrylamide–urea sequencing gel. The products of Maxam–Gilbert ‘G + A’ and ‘A > C’ cleavage reactions of the same fragment were used as markers. Positions with numbers according to Rott et al. (1988) and lines for the protected regions are indicated. The right panel is a longer run of the samples analysed in the left panel. Lanes 1 to 3, controls, no protein added.
DNA binding of BFDV large T antigen

Fig. 8. Summary of DNase I protection results in T antigen/non-coding DNA binding. Hatched bars (A, B, C and D) indicate the nucleotides protected by the large T antigen from DNase I cleavage. The top or bottom lines (a to l) represent 12 putative DNA-binding sites (also see Fig. 9), whereas arrows indicate DNase I hypersensitive sites. The structural organization of the BFDV non-coding region, including the P<sub>F</sub> TATA box, early mRNA cap sites and T antigen initiator codon as well as the 45 bp tandem repeats, is shown together with the complete DNA sequence. The numbers above the sequences are according to Rott <i>et al.</i> (1988).

Repeat domain II, which region C extends equivalently within repeated domain I. Although repeat domains I and II contain sequences that are very similar, the extent of protection from DNase I cleavage at repeat domain II is somewhat greater than at repeat domain I, which agrees well with the slightly stronger binding for domain II as observed in the DNA immunoprecipitation binding experiments. Region D is located outside of repeat domain I and covers a region near the early promoter TATA box. DNA footprinting assays were performed with lower concentrations of T antigen. However, intermediate levels of protection were not observed (data not shown). This might suggest that the T antigen binds to DNA cooperatively or as a multimer complex.

Discussion

As a result of the determination of the early and late mRNA 5' ends and the corresponding early and late promoters (D. Luo, H. Müller & G. Hobom, unpublished data; Stoll <i>et al.,</i> 1993), the viral non-coding region controlling transcription can be defined much more precisely. It extends over 289 bp in between the T antigen and agnoprotein initiator codons, or over 233 bp
in between the early and late mRNA 5' ends, with a core region of only 179 bp upstream of the two early and late promoter TATA boxes. This region, therefore, is considerably smaller than in SV40 or other polyomaviruses, and much smaller than initially predicted for BFDV (Rott et al., 1988).

The small size of the BFDV non-coding region, compared with that of other polyomaviruses, indicates that either this virus must have a more simply organized regulatory system, or that it is spatially more tightly interlinked than in other cases (SV40 and Py). The DNA binding and footprinting studies performed with the purified T antigen have shown that the protein-binding sites are indeed confined to what has been defined above as the core region and do not extend into neighbouring regions. On the other hand, these studies show that essentially all of this region except approximately 50 bp (positions 180 to 230) is covered in the T antigen footprint. In detail we found that the primary binding site is related to the region containing a 45 bp major repeat sequence by restriction enzyme NsiI recognition sites are directly involved in the overall binding of the large T antigen.

DNase I footprint analyses demonstrated four non-contiguous regions of DNase I protection in the non-coding region. These regions of T antigen binding are in agreement with the conclusions drawn from DNA-binding immunoprecipitation assays. Surprisingly the major 45 bp tandem repeat sequences in BFDV, repeat domains I and II (26 to 72 nt, and 77 to 124 nt upstream of the TATA box of early promoter P\(_e\)), are directly involved in binding the BFDV large T antigen. This differs from the organization in the SV40 non-coding region, since the major repeat sequences, similar in length and located at an equivalent position, do not bind SV40 T antigen, but act as a host protein-binding enhancer region in mammalian SV40 DNA. Besides these two primary binding sites for the T antigen, two weaker and probably secondary binding sites have been observed in these studies, one immediately adjacent to and extending into the P\(_e\) TATA box, the other at the right-hand edge of repeat sequence II. Based on the DNA sequences covered by the T antigen in the two palindromic repeat sequences and in the two additional sites a total of 12 binding sequences can be defined that constitute a consensus sequence provisionally given in Fig. 9. At present these BFDV T antigen-binding sites are less strictly defined than the corresponding binding sites in SV40 (DeLucia et al., 1983; Tegtmeyer et al., 1983; Tenen et al., 1983) or Py (Gaudry et al., 1981; Pomerantz et al., 1983; Cowie & Kamen, 1984).

Although the protein segment with which BFDV large T antigen binds to DNA is not yet known, partial analogy to SV40 large T antigen and its genetic organization, and the presence of two potential zinc finger domains (AA85, Cys-X\(_2\)-Cys-X\(_{15}\)-His-X\(_5\)-His; AA225, Cys-X\(_2\)-Cys-XCY\(_{13}\)-His-X\(_5\)-His) in the corresponding region (Rott et al., 1988), suggests that this region of the protein sequence may be directly involved in specific binding to DNA (Loebel et al., 1989; Bergqvist et al., 1990a; Höss et al., 1990a). Structural analysis of the zinc finger protein–DNA complex indicated that most of the contacts involve the guanine-rich strand of the DNA (Pavletich & Pabo, 1991).

The differences in the arrangement of binding sequences between avian polyomavirus and its mammalian counterparts imply a different strategy of interaction between the T antigen and the viral DNA, and may also indicate different modes of interaction with regulatory host factors, e.g. p53 and pRB. It has been shown that wild-type and not mutant p53 interacts with the SV40 T antigen (Weinberg, 1991) and that it also binds strongly to sequences adjacent to the SV40 replication origin, which results in prevention of the SV40 T antigen from interacting with its binding sites (Barponetti et al., 1991). Furthermore, tumour suppressor gene product pRB (retinoblastoma) forms complexes with a variety of viral oncoproteins including the SV40 large T antigen (Weinberg, 1991). The BFDV large T antigen appears to contain a potential pRB-binding site in comparison with other viral binding sequences, so that it may indeed interact with pRB (H. Müller, unpublished data). However no interaction has yet been observed with p53 by testing for co-immunoprecipitation (H. Müller, unpublished data).

Although many reports describe the failure of the baculovirus system to remove intron sequences from premRNA molecules, Jeang et al. (1987) have reported the construction of a recombinant baculovirus containing the coding sequence from the SV40 early region and show that the small t mRNA splice reaction was
considerably favoured over the large T alternative splice reaction in insect cells. However, in recombinant virus Ac-HL644 which contains a genomic copy of the early region from BFDV, only small t antigen is synthesized in significant amounts, and there is no detectable accumulation of large T antigen and correspondingly no large T antigen expressed in insect cells may differ from that occurring in wild-type BFDV-infected cells, and may at least in part be due to different phosphorylation patterns (H. Müller, unpublished). The biological significance of the phosphorylation of the BFDV large T antigen is not yet known. However, it is known that DNA binding as well as DNA replication functions of SV40 large T antigen are strongly influenced by a complex pattern of protein phosphorylations (Prives, 1990; Höss et al., 1990b). This and other modifications remain to be analysed in this regard for the BFDV T antigen.

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