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ANALYSIS OF HAMSTER LYMPHOMAS FOR THE PRESENCE OF HAMSTER PAPOVAVIRUS DNA

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Summary. — The hamster papovavirus (HaPV) is a polyomavirus isolated from skin epitheliomas arising spontaneously in young Syrian hamsters. It can induce lymphomas and leukaemias in newborn hamsters. Although no virus particles are detectable by electron microscopy, high amounts of monomeric and oligomeric forms of extrachromosomal HaPV DNA molecules are found in the lymphoma cells. These molecules display deletions of about 300 nucleotides in length. Their role in the lymphoma induction is discussed.

Key words: Hamster papovavirus; lymphomatogenesis; deletion mutants; Southern blotting; molecular cloning

Introduction

The hamster papovavirus (HaPV) has been classified as a member of the polyomavirus group (Vogel *et al.*, 1986). The virus was originally isolated from multiple skin tumours of the Syrian hamster by Graffi *et al.* (1967, 1968). In every primary epithelioma very large numbers of virus particles fill the nuclei of cells in the keratinized layer. The tumours are transplantable but no virus can be detected in the transplanted tumours (Graffi *et al.*, 1970). The virus is morphologically indistinguishable from polyoma virus or SV40. The HaPV-DNA which can be isolated from purified virions is a double stranded circular molecule with a molecular weight of about 3.1×10^6 daltons (Böttger *et al.*, 1971; Scherneck *et al.*, 1979). The viral genome has been cloned and sequenced recently and consists of 5368 base pairs (Delmas *et al.*, 1985). The genomic organization deduced from the open reading frames is of the polyoma type with the two strands coding in the opposite direction from a noncoding region. The HaPV is after polyoma virus the second papovavirus coding for a middle T antigen. The virus and the (cloned) viral DNA can immortalize primary cells and transform established cell lines from rodent origin (Delmas *et al.*, 1985). The HaPV (DNA) can also induce lymphomas

and leukaemias after subcutaneous inoculation of newborn animals from a hamster strain different from the strain showing spontaneous epitheliomas. This strain has a very low incidence of spontaneous tumours, especially lymphomas and leukaemias (Graffi *et al.*, 1969); the incidence is high (30—80%) and the latency short (4—8 weeks). Virions have not been detected in the lymphoma cells by electron microscopy. We have anlysed several lymphomas induced after subcutaneous inoculation of newborn hamsters with HaPV virions and DNA, respectively, for the presence of HaPV DNA. In this report we can demonstrate that the tumour cells contain large amounts of viral DNA and they actively replicate the viral DNA as extrachromosomal molecules.

Materials and Methods

Lymphoma induction. Doses of 0.2 ml of a virus suspension purified by caesium chloride centrifugation from hair follicle tumours (Scherneck *et al.*, 1979) were inoculated subcutaneously into newborn hamsters of a colony largely free of spontaneous tumours.

DNA isolation. a) Viral DNA: The HaPV DNA was extracted from virus preparations purified from pools of skin tumours as previously described (Scherneck *et al.*, 1979). b) Plasmid DNAs: Plasmid DNAs were propagated in *E. coli HB101* and purified by conventional methods. c) Total cellular *DNA*: Total cellular DNA was extracted from homogenized fresh tissues according to Gross-Bellard *et al.* (1973).

Molecular cloning of HaPV genomes from lymphoma DNA. Low molecular weight DNA was extracted from 500 μg of total lymphoma DNA according to Hirt (1967) and cloned at the *BamHI* sites of dephosphorylated *pML2* plasmids. The HaPV DNA insert were selected by colony hybridization with a ^{32}P-labelled nick-translated viral HaPV probe.

Restriction endonuclease cleavage. Radiolabelling, blotting and hybridization of the DNAs. The restriction enzymes were from Boehringer or ZIMET (Jena) and used as recommended by the suppliers. Total cellular, viral and cloned DNAs were separated by electrophoresis through 1% (cellular DNA) or 1.2% (w/v) agarose gel slabs as previously described (Zimmermann *et al.*, 1984). Radiolabelling, blotting and hybridization of the DNAs followed with minor modifications the protocols of Rigby *et al.* (1977); Southern *et al.* (1975) and Wahl *et al.* (1979). In reconstruction experiments our hybridization assay could detect from 0.5 to 0.2 genome equivalents per cell, according to the specific activity of the probes.

Electron microscopy. The methods used for detection of virus particles in lymphoma tissues are described by Graffi *et al.* (1970). Heteroduplexes were prepared and examined according to Davis *et al.* (1971). DNAs were observed with a Philips EM400 electron microscope and length measurements were done directly from electron plates using a Zeiss Documator 2GL. Distribution histograms were constructed with the help of a program package installed on a Hewlett-Packard calculator (HP 9845 B).

Results

Induction of lymphomas

In 1969 Graffi and coworkers demonstrated that subcutaneous inoculation of HaPV virions or HaPV DNA, isolated from primary epitheliomas, could induce lymphoma and leukaemia in Syrian hamsters. It is of importance that these tumours were only induced in a special hamster colony largely free of spontaneous lymphomas and skin epitheliomas (Graffi *et al.*, 1969).

We have confirmed Graffi's experiments in several independent assays. The incidence of lymphoma induction varied between 50 and 70% of the

injected hamsters and the latency was between 4 and 8 weeks. The tumours originated mostly in the liver and they were histologically characterized as B lymphomas. More than 20 lymphomas were screened for virus particles. In none of them we have succeeded in detecting either papovaviruses or C-type virus particles. The latter observation is in contrast with earlier data by Graffi *et al.* (1970) who demonstrated in the lymphomas a small number of C-type virus particles resembling murine leukaemia viruses.

Detection and characterization of HaPV DNA in the lymphoma cells

Although no virus particles were detectable in the lymphoma cells, we examined the total DNA prepared from lymphoma tissues for HaPV DNA sequences. The cellular DNA, either uncleaved or digested with the single cut enzyme *Bam HI* was fractionated by agarose gel electrophoresis, transferred to nitrocellulose paper and hybridized to a ^{32}P-labelled HaPV DNA probe. As a control HaPV DNA was subjected to the same procedure. As shown in Fig. 3 and Fig. 5 large amounts of HaPV DNA could be detected in the tumours. In some lymphomas more than 1000 copies/cell were present. The electrophoretic pattern obtained with the uncut DNA of 3 independent tumours clearly shows that the viral DNA exists as free monomeric molecules which can be linearized by digestion with *Bam HI* (Fig. 5). In some other tumours the HaPV is present in the form of high molecular weight oligomers which can be cleaved to unit length linear molecules by *Bam HI* (Fig. 3). Overexposure of the *Bam HI* digest does not reveal extra bands which could indicate the presence of integrated genomes. However, the high number of DNA copies present in the lymphoma cells could mask the existence of a small number of integrated molecules. The tendency for integration seems to be increased by passaging a tumour several times in animals. Fig. 7 shows Southern blotting analysis of total DNA, isolated from lymphoma 773 after passage 2 and passage 28, respectively, in hamsters. After passage 2 the great majority of the uncut HaPV can be linearized with *Bam HI* and only a faint extra band could indicate the existence of integrated genomes. On the other hand undigested DNA isolated from Ly773 after passage 28 stayed in the high molecular weight region. When digested with *Bgl II*, an enzyme which does not cut HaPV DNA, the viral DNA remains as a discrete band in the high molecular weight range, suggesting an integration at a definite site within the host genome. The digestion with the single site enzyme *Bam HI* shows a pattern of two heavely and one weakly labelled band. One of the heavy bands represented complete viral DNA molecules, the other presumably are the junction fragments. These cleavage patterns suggest that the HaPV DNA is integrated into the host genome, probably as a tandem repeat. A similar conclusion has been reached on the status of HaPV DNA in immortilized primary cells and transformed rodent cell lines (Delmas *et al.*, 1985).

The structure of the extrachromosomal viral DNA was further characterized by restriction analysis. The total DNAs of two lymphomas, Ly1 and Ly3, were digested with 5 different restriction enzymes. As a control, HaPV DNA prepared from a virus pool which was isolated from primary epitheliomas,

was subjected to the same enzymes. Southern blot analysis and hybridization of these DNAs to ^{32}P-labelled HaPV DNA showed no major differences in the electrophoretic pattern of the respective DNA fragments (Fig. 1). For a more detailed analysis we have cloned out from an individual total lymphoma DNA (Ly3), after high salt precipitation of the high molecular weight DNA, HaPV DNA molecules. The soluble DNA were cleaved with *Bam HI* and cloned at the *Bam HI* site of a *p*ML 2 vector. One of the HaPV DNA containing recombinant plasmids which is representative for the clones isolated from the tumour, was compared with the cloned HaPV DNA (puc13-HaPV) isolated from primary epitheliomas. The restriction analysis provide a precise approach for this study. The *Bam HI* + *Eco RI* digestion of free viral DNA is expected to give rise to five restriction fragments obtained by the digestion of the cloned HaPV DNA (puc13-HaPV) (Fig. 2b). These five bands can be observed after *Bam HI* + *Eco RI* digestion of the HaPV DNA cloned out from tumour, indeed. However, fragments B and E show electrophoretic mobilities which differ from those of the respective puc13-HaPV fragments (Fig. 2d) To localize these differences more precisely, we constructed heteroduplexes between the DNAs cloned from the lymphoma (Ly. 3.3 DNA) and puc13-HaPV. In this study the HaPV DNAs were cut out from their plasmid vector at the *Bam HI* cloning site and hybridized under restrictive conditions (50% formamide) requiring at least 80 to 85% of homology for stable duplex formation. The hybrid presented in Fig. 6 shows a rather uniform structure made of a duplexed region and a single stranded DNA loop, the latter about 300 nucleotides long. The respective identity of the two molecules in the duplex was obtained after treatment of the Ly3.3 DNA with a second single site enzyme (*HpaI*) which localize the deletion near the origin of replication of the Ly3.3 genome (data not shown here).

In a series of experiments we have tested healthy organs from lymphoma bearing animals for the presence of HaPV DNA. None of them shows detectable amounts of viral DNA (Fig. 4).

Discussion

The HaPV shows a unique tumour spectrum among the group of polyomaviruses. Originally the virus was identified by Graffi *et al.* (1967) as the causative agents for multiple skin epitheliomas of the Syrian hamster, a tumour type which is most common for papilloma viruses and reflects the capacity of the virus to infect and replicate in keratinocytes. Coggin *et al.* (1983, 1985) described a very similar virus associated with skin epithelioma of the Syrian hamster and presented evidence that the virus is the aetiologic agent of the tumour. Subcutaneous injection of HaPV virions or -DNA, on the other hand, result in the induction of lymphomas in high incidence and short latency periods in newborn hamster. However, the occurrence of lymphomas is dependent from a special strain of Syrian hamsters which is largely free of spontaneous tumours. Two other papovaviruses share this lymphotropism: SV40 can induce a large spectrum of haematopoietic tumours

when injected intravenously in the Syrian hamster (Diamandopoulos 1972) and the lymphotropic papovavirus (LPV) was isolated from a monkey B lymphoid cell line (zur Hausen and Gissmann, 1979). In the present study we have analysed more than 30 lymphomas for the presence of HaPV specific DNA/or virions. In all of these lymphomas tested so far we never found HaPV virion particles but large amounts of HaPV DNA. The viral DNA was found to exist in the cells almost exclusively as extrachromosomal molecules, whenever large amount of viral DNA not definitely excludes the presence of single copies of integrated DNA. Integrated HaPV DNA can clearly be demonstrated in such tumours which were passaged *in vivo* several times. In this respect the situation is similar to that described for papillomaviruses (Gissmann, 1984). On the other hand, the HaPV DNA is stable integrated in the genome of several in vitro transformed rodent cells (Delmas *et al.*, 1985); this is the general situation in polyomavirus transformation (Tooze, 1980).

The main fraction of the viral DNAs found in the tumours were analyzed by restriction endonuclease cleavage and consist of deleted DNA molecules. A more detailed analysis of representative viral DNA molecules which were cloned out a tumour show that these deletions are about 300 nucleotides in length and located near the origin of replication on the viral genome. Preliminary results obtained from the characterization of further cloned HaPV--DNA molecules isolated from 3 independent lymphomas confirmed this statement (Scherneck *et al.*, unpublished results).

The regular presence of particular HaPV DNA in the lymphomas but not in the healthy organs of lymphoma bearing animals does not prove a causative role in cancer. Additional information about the molecular structure of the deletion mutants, the mechanisms for their generation and selection is required for further clarification. Finally a more detailed knowledge of the early viral functions but also mRNA and protein identification experiments are necessary to understand how the HaPV participate in lymphoma development.

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Legends to the Figures (*Plates XVII–XIX*):

Fig. 1. Southern blot analysis of total genomic DNA from lymphoma tissues Ly 1 and Ly 3. The DNAs (10 μg) were digested with restriction endonucleases *Bam HI* (A, F); *Eco RI* (B,G); *Hind III* (C, H); *Kpn I* (D, I) and *Hae II* (E, K), electrophoresed in a 1% agarose gel and stained with ethidium bromide. As a reference, viral DNA (0.2 μg), isolated from skin epithelioma were digested with the respective enzymes (L — P) (upper part). The blot was probed with a nick-translated ^{32}P-viral DNA (lower part, a — p).

Fig. 2. Restriction patterns of HaPV DNA cloned from total Ly 3 DNA with the plasmid pML 2 (Ly 3.3) and cloned HaPV DNA isolated from skin epitheliomas (*puc13-HPV*). The DNAs were digested with *Bam HI* (a, c) or *Bam HI* + *Eco RI* (b, d) and electrophoresed on a 1.2% agarose gel. The blot was probed with a nick-translated ^{32}P;puc13-HaPV DNA.

Figs. 3 and 5. Southern blot analysis of total genomic DNAs from tissues of different lymphomas. The DNAs were used undigested (a, b, c, d, a, c, e) or digested with *Bam HI* (a′, b′, c′, d′; b, d, f) and electrophoresed on a 1% agarose gel. The blot was probed with a nick-translated ^{32}P viral DNA.

Fig. 4. Southern blot analysis of total genomic DNA of tissues from different healthy organs of hamsters suffering from lymphomas. The undigested or Bam HI digested DNAs (20 μg) were

electrophoresed on 1% agarose gels. The blot was probed with a nick-translated ^{32}P viral DNA; (a) liver uncleaved, (b) liver *Bam HI* clevad; (c) kidney uncleaved, (d) kidney *Bam HI* cleaved (e) spleen uncleaved, (f) spleen *Bam HI* cleaved; (g) thymus uncleaved, (h) thymus *Bam HI* cleaved.

The viral DNA (k) representing 10^3 copies and the total Ly 1 and 3 DNAs (i, j) provide the markers.

Fig. 6. Heteroduplexes between the cloned HaPV DNA, isolated from skin epitheliomas, and Ly 3.3 DNA. The recombinant DNA molecules were digested with *Bam HI* and hybridized under high stringency conditions (50% formamide). The arrow indicates the nonhomologous region within the viral genomes. The bar marker represents 1 kb.

Fig. 7. Southern blot analysis of total DNA isolated from in vivo passaged lymphoma tissues (Ly 773). The lymphoma was induced after inoculation of HaPV virions in a newborn hamster. Parts of the tumour were removed, homogenized and mixed with 4 parts of PBS. 1 ml of this mixture was inoculated subcutaneously into 3—4 weeks old hamsters. Total DNA (10 μg) isolated from tumours arising after passage No. 2 (P 2) and passage No. 28 (P 28) was either non-cut or digested with *Bam HI* or *Bgl II* and electrophoresed on a 1% agarose gel. The HaPV DNA isolated from skin epithelioma provide the size marker. The blot was probed with a nick-translated ^{32}P viral DNA.