Presence of integrated DNA sequences of adeno-associated virus type 2 in four cell lines of human embryonic origin

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The human helper virus-dependent parvovirus adeno-associated virus (AAV) has been found in human female genital tissues including material from first trimester miscarriage. In the latter case, AAV type 2 (AAV-2) DNA and viral proteins were detected mainly in the trophoblast cell layer of placenta. In this report, we present evidence that AAV DNA is also present in established human trophoblast cell lines (JEG-3, JAr, BeWo) and in the human amnion cell line FL. In cells of these lines, AAV-2 DNA could be detected both by PCR and Southern blot analysis. Restriction enzyme analysis indicated that AAV DNA was integrated into the host cell genome. Although the cell lines supported AAV replication when infected with AAV-2 and adenovirus type 2 (Ad2) as a helper virus, super-infection with Ad2 alone did not induce replication of AAV DNA, i.e. it failed to rescue AAV from its integrated state. This is probably due to rearrangements within the integrated AAV genome. The presence of AAV DNA in cells derived from human embryonic tissue corroborates the suggestion that human embryonic tissue may be one of the targets of AAV infection.

The human helper virus-dependent parvovirus adeno-associated virus (AAV) is thought to be non-pathogenic (Berns & Bohenzky, 1987) but to exert oncosuppressive activity (Schlehofer, 1994). In light of this, and because of its ability to integrate its genome into the host cell genome at preferential sites (Kotin & Berns, 1989; Kotin et al., 1990; Walz & Schlehofer, 1992), AAV has attracted much interest as a gene transduction vector (Muzyczka, 1992; Flotte & Carter, 1995). In natural infections in humans, AAV has been identified in female genital tissue and in material from spontaneous abortion (Tobiasch et al., 1994; Han et al., 1996; Friedman-Einat et al., 1997; Walz et al., 1997). In samples from first trimester miscarriage, DNA and proteins of AAV were detected predominantly in trophoblast cells (Tobiasch et al., 1994). This finding prompted us to analyse the choriocarcinoma-derived human trophoblast cell lines BeWo (Pattillo & Gey, 1968), JEG-3 (Kohler & Bridson, 1971) and JAr (Pattillo et al., 1971), as well as the human amnion-derived FL cell line (Fogh & Lund, 1957), for AAV. Analysis by PCR using primers amplifying sequences of AAV (Tobiasch et al., 1994) indicated the presence of DNA of AAV type 2 (AAV-2) within the human trophoblast cell lines (Fig. 1a). These results could be confirmed using primers proposed by Han et al. (1996) (data not shown). In addition, Southern blot analysis revealed AAV-2 sequences migrating with high molecular mass cellular DNA, when DNA digested with restriction enzymes that did not cut within the AAV-2 DNA sequence, typically sized AAV-2 DNA fragments (Srivastava et al., 1983) were observed (Fig. 1b). However, transcription from sequences of AAV-2 could not be detected by RT–PCR or Northern blot analysis (data not shown).

In order to test whether AAV-2 DNA present in trophoblast cells can be rescued, we infected BeWo, JEG-3, and JAr cells with adenovirus type 2 (Ad2). As shown in Fig. 2, no viral DNA replication nor production of viral particles could be achieved by infection of these cells with helper virus, in contrast to HA16 control cells which contain integrated and rescuable AAV-2 DNA (Walz & Schlehofer, 1992). To ensure that these cell lines can be productively infected with adenovirus and/or AAV, cells of the BeWo, JEG-3 and JAr lines were infected with Ad2 (m.o.i. of 5 p.f.u. per cell) and co-infected with AAV-2 (m.o.i. of 10 p.f.u. per cell). Fig. 2 shows that AAV-2 replicates in cells co-infected with AAV-2 and Ad2. This indicates that a non-functional AAV-2 DNA sequence exists within trophoblast cell lines which is probably due to rearrangements of AAV DNA. Indeed, in JEG-3 cells, a

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point mutation was found at base position number 499. This specific mutation has been reported to suppress Rep activity (Hermonat et al., 1984).

In JAr cells, it was not possible to amplify AAV DNA sequences when using the rep specific primers (p1, nt 960–941; p2, nt 305–320) described by Samulski et al. (1991) (un-
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Fig. 3. Presence of integrated AAV DNA sequences within the genome of FL cells. (a) Analysis of genomic DNA from FL cells. Cellular DNA [10 µg from FL cells co-infected with Ad2 and AAV-2 (FL+AAV+Ad2), 10 µg from HA16 cells and 30 µg from FL cells] was digested with restriction enzyme BglII, which does not cut within the AAV-2 genome. DNA was separated and blotted as indicated in Fig. 1 (b). The membrane was hybridized as in Fig. 1 (b) except that undigested pTAV2 was used. The right panel shows the same HA16 and FL lanes after overexposure (72 h). Molecular size markers (Mk) are indicated. (b) Lack of rescue of AAV-2 DNA after adenovirus infection of FL cells. Cells were infected with either PBS (mock infection) or Ad2 (m.o.i. of 5 p.f.u. per cell) and harvested after complete cytopathogenic effect (72 h.p.i.). Cells were then treated and filters were hybridized with the AAV-2 probe as described in Fig. 2 (left two panels) or with an α-32P-labelled Ad2 probe (E1α insert, courtesy of F. Rosl, DKFZ/ATV, Heidelberg, Germany) (right two panels). HA16 and HeLa cells are used as positive and negative controls for AAV rescue after Ad2 infection. (c) Detection by PCR analysis of HPV-18 (upper panel) and AAV-2 (lower panel) DNA in five different single-cell clones of FL cells (numbered c19 to c23). PCR conditions were as described by Malhomme et al. (1997) for the detection of HPV-18 DNA and as in Fig. 1 (a) for the detection of AAV-2. Amplification products were blotted onto nylon membranes after separation on agarose gel and the corresponding blots were hybridized with specific HPV-18 (upper panel) and AAV-2 (lower panel) oligonucleotide probes Malhomme et al., 1997). Clones c19, c20 and c23 contain only HPV-18 DNA, clone c22 contains only AAV-2 DNA and clone c21 contains both HPV-18 and AAV-2 DNA.

Published observations). These preliminary data suggest a deletion within the rep coding region of the AAV DNA.

Similarly, another human cell line (FL) of embryonic origin was found to contain AAV DNA sequences by PCR and Southern blot analysis (Fig. 3a). Also, the AAV genome could not be rescued in these cells after superinfection with helper virus (Fig. 3b), although these cells were permissive for AAV DNA amplification after superinfection with Ad2 (Fig. 3a, first lane). This cell line, used in many virology laboratories, is derived from human amnion tissue and displays epithelial-like morphology (Fogh & Lund, 1957). FL cells are known to exhibit HeLa cell markers and are suspected to be contaminated by HeLa cells (ATCC catalogue information). Since HeLa cells contain sequences of human papillomavirus type 18 (HPV-18), we analysed clonal isolates of these cells for the presence of AAV-2 and HPV-18. Using specific primers and probes, we found that the FL cell line is indeed a mixture of cells. HPV-18 DNA sequences were observed in 16 out of 21 single cell clones. Eleven HPV-positive clones also contained AAV-2 DNA sequences. A minority of clones contained only AAV-2 DNA (two out of 21) or were negative for the presence of both viral DNA sequences (Fig. 3c).

AAV-2 has been repeatedly found with notable frequency in the human genital tract (Tobisch et al., 1994; Han et al., 1996; Friedman-Einat et al., 1997; Walz et al., 1997). The data presented herein show, for the first time, the presence of AAV-2 DNA integrated within the cellular genome of established human cell lines of choriocarcinoma (JAr, JEG-3 and BeWo) or...
amnion (FL) origin. The AAV DNA sequences were found repeatedly, either by PCR or Southern blot, in cells from different suppliers or laboratories (see Acknowledgements) thus excluding the possibility of recent contamination in our laboratory.

In contrast to other cell lines containing integrated AAV DNA after experimental infection or transfection (Laughlin et al., 1986; Walz & Schlehofer, 1992), AAV could not be rescued from JAr, JEG-3, BeWo or FL cells after superinfection with Ad2. The fact that these cell lines supported replication of AAV when co-infected with AAV-2 and Ad2, indicates that the integrated AAV-2 sequences underwent molecular modifications preventing excision and replication by helper viruses. This is substantiated by our results of some rearrangements within the AAV DNA integrated in the genome of trophoblast cells.

After digestion of the cellular DNA of trophoblast cells, the fragments hybridizing to AAV probes revealed slightly different mobilities, suggesting different integration sites in different cells. However, our results do not indicate that multiple integration of rearranged and/or tandemly organized AAV-2 DNA molecules has occurred in naturally infected JAr, JEG-3 and BeWo cells. In previously described experimental systems leading to AAV-2 DNA integration, virus sequences were found integrated predominantly within a cellular sequence named AAVS1 (Linden et al., 1996). Since it is unlikely that the AAVS1 sequence is organized differently in cells from placental origin (Kotin et al., 1990), it is conceivable that the unique, high molecular mass, AAV-2 DNA band observed, even if slightly smaller in BeWo cells, may represent a single integration site.

On the other hand, the BglII restriction pattern of DNA from FL cells (Fig. 3a) is reminiscent of that observed previously with experimentally infected cells such as HeLa, KB and Detroit-6. These cells all exhibit HeLa markers [KB, ATCC information; Detroit-6 cells, Mikhailova et al. (1977)] which are also present in FL cells (ATCC information, our results). This might suggest that integration of multiple copies of AAV-2 DNA in multiple integration sites is a particular feature of HeLa or HeLa-contaminated cell lines.

To answer these questions, analysis of the integration pattern of AAV-2 sequences in trophoblast cells as well as cloning of integrated molecules are currently under investigation in our laboratory.

In view of the oncosuppressive activity of AAV, it was surprising to find AAV-2 DNA sequences ‘naturally’ integrated within the genome of tumour (choriocarcinoma) cells. Possibly, modified (unrescuable) AAV-2 DNA sequences do not express oncosuppressive activity.

Altogether, our data, as well as previous reports (e.g. Tobiasch et al., 1994), indicate that human embryonic cells might be a target of natural AAV infection, although we cannot exclude an infection with AAV in the very early steps of the establishment of JEG-3, JAr, BeWo and FL cells. However, it seems unlikely that these cells, which were obtained at different times and in different laboratories, could have been the only AAV-contaminated cell lines among the multitude of other human embryonic cells cultured in vitro; for instance, the human Tera-1 cell line of teratocarcinoma origin was negative for the presence of AAV-2 DNA sequences (T. Burguete, unpublished results). Nonetheless, it seems important to note that some human embryonic cell lines which are used in many laboratories are persistently infected with AAV-2.

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