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Research Article

Localization of integrated adenovirus DNA in the hamster genome

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Abstract. Adenovirus DNA integrated into the genomes of adenovirus-transformed hamster cells or of adenovirus type 12 (Ad12)-induced hamster tumor cells can be located at many different chromosomal sites. This raises the question as to whether distinct isochores of the hamster cell genome might be more accessible to recombination with adenovirus DNA. In Ad12- or Ad2-transformed hamster cell lines, and in Ad12 revertants, the investigated integrated viral DNA sequences were assigned to isochore families by analyzing DNA fractions from preparative CsCl density gradients for their buoyant densities (and, therefore, GC levels) and for the presence of viral DNA. Adenovirus DNA sequences were found in different isochores, which did not generally match the base composition of viral sequences. This is in apparent contrast to what was previously observed with retroviral integration. However, in cell lines carried in culture for many years, the viral DNA sequences might have been transposed to different isochore positions, since the host sequences flanking the viral DNA appear to have been conserved.

Key words. Foreign DNA integration; adenovirus-transformed cells; isochores in mammalian DNA; recombination site.

The integration of adenovirus DNA into the hamster genome has been studied using adenovirus type 12 (Ad12)- or Ad2-transformed hamster cell lines and Ad12-induced hamster tumors [1]. Viral DNA has been exclusively found in an integrated form – free viral DNA has never been observed. Analyses of more than 100 different transformed cell lines and Ad12-induced tumors have not revealed evidence for a specific chromosomal site or for unique host cellular DNA sequences for viral DNA insertion. Patchy homologies between cellular and viral sequences at, or close to, the junction sites have frequently been observed over stretches up to 40 nucleotides long [2]. In both hamster cells devoid of viral DNA integrates and in the transformed cells, the identified preinsertion sites have often been found to be transcriptionally active. These host DNA segments might have a higher propensity to recombine with foreign DNA such as adenovirus DNA. Here, we turned our attention to the isochore organization of mammalian genomes in the search for clues as to the nature of sites of insertional recombination.

Mammalian genomes are mosaics of isochores, DNA segments of >300 kb with fairly homogeneous base composition which belong to a small number of families covering a wide spectrum of GC levels [3, 4]. The gene concentration increases from a low average level in L isochores to a 20-fold-higher level in H3 isochores [5, 6]. The chro-

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matin structures are 'open' in the gene-rich H isochores and 'closed' in the gene-poor isochores [7, 8]. The open chromatin structures are more accessible to DNase cleavage [9], show a wider spacing of nucleosomes, absence of histone H1 and acetylation of histones H3 and H4 [10]. Replication in the cell cycle is late in GC-poor and early in GC-rich isochores [8]. Transcription is clustered in GC-rich regions, but scattered in GC-poor regions, and the GC-richest, transcriptionally active compartments in the genome are also the most recombinogenic ones [4].

The development of methods to fractionate DNA at high resolution [11, 12] allowed proviral sequences to be localized [13]. Retroviral DNA integration was stable in regions of the host genome with a compositional match with the viral sequences [14]. Similar results were obtained for the hepatitis B virus genome in human cell lines [15]. In all cases, 'isopycnic' integration (namely, integration with a compositional match) was accompanied by transcription of viral sequences, whereas non-isopycnic integration was not.

In this report, we investigated the localization of integrated adenovirus DNAs in several Ad2- and Ad12-transformed hamster cell lines.

Materials and methods

Cells and viruses

The origins of and the procedures for propagating the Ad12-transformed hamster cell lines HA12/7 [16] and T637 [17], the Ad2-transformed hamster cell line HE5 [18], and the morphological revertants TR2 and TR12 of the Ad12-transformed hamster line T637 have been presented elsewhere [19–21]. Ad2 and Ad12 were propagated and purified, and their DNAs were isolated as outlined earlier [22]. Table 1 summarizes the properties of the cell lines used in this work.

Extraction of cellular DNA

Confluent cells growing on 175-cm^2 surface areas were washed twice with Tris-saline and detached from the plastic support by treatment with Tris-saline/EDTA. Subsequently, cells were washed again, resuspended in proteinase K-buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 200 mM NaCl), and were then incubated with proteinase K (Merck) and 1% SDS at 55 °C for 16 h. Cellular DNA was further purified by several phenol-chloroform extractions. The upper phase was separated by a silicone layer (Baysilone) from the lower phase and interphase. Thus, the aqueous phase could be decanted and pipetting was avoided. DNA was incubated with RNase A for several hours and was again purified by phenol-chloroform extraction. Subsequently, the DNA was precipitated with ethanol and resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE). The sizes of the prepared DNA fragments were determined by pulse-field electrophoresis (BioRad Chef Mapper) and ranged between 150 and 300 kb.

Preparative buoyant density gradient centrifugation

About 10–30 μg DNA from the different cell lines was fractionated by preparative equilibrium sedimentation in CsCl density gradients in TE [23, 24] with a refractive

Table 1. Ad2- and Ad12-transformed cell lines and Ad12-induced tumor cell lines.

Name of cell line or tumor	Virus	Mode of transformation or tumor induction	Copy number per cell	State of integrated DNA	Transcriptional activity of preinsertion sequence	References
T637	Ad12	BHK cells, in vitro	15–20	'intact' colinear, right end inverted (in some copies)	no	17, 21
TR12	Ad12	BHK cells, in vitro; revertant cell line of T637	1–2	one Ad12 molecule and a part of the right half preserved	no	19, 20
TR2	Ad12	BHK cells, in vitro; revertant cell line of T637	<1	right-hand portion preserved	no	19, 20
HA12/7	Ad12	primary hamster embryo cells, transformed in vitro	3	intact, colinear	yes	16
CLAC1	Ad12	injection of CsCl-purified Ad12 into CLAC inbred hamsters; establishment of cell line	(12)		yes	31, 38
HE5	UV-inactivated Ad2	LSH primary embryo cells, transformed in vitro	2–3	fragmented	yes	2, 18

Table 2. Positions and sequences of primer pairs used in PCR reactions.

Primer name	Position	Sequence coordinates	Sequence
L1	*Pst*I-L fragment of Ad12	nt 33028–33046	5′-cca cac ctt cgc gca-3′
L2	*Pst*I-L fragment of Ad12	nt 33398–33416	5′-cac ccg ttt gcc agg cat c-3′
F5-1	preinsertion site	nt 873–893	5′-gct gtg cca gtc cct tac ag-3′
F5-2	preinsertion site	nt 1072–1093	5′-gat gtc aga tac atc cag gta g-3′
ad2-1	left terminus of the Ad2 genome	nt 476–495	5′-ccg gtg agt tcc tca aga gg-3′
ad2-2	left terminus of the Ad2 genome	nt 899–879	5′-ttt ggc ata gaa acc gga ccc-3′
E4-1	E4 region of the Ad12 genome	nt 32931–32961	5′-gcc atc cgc agg cag tac ttc-3′
E4-2	E4 region of the Ad12 genome	nt 33359–33337	5’-aag cat gtt tca gag gtc gct tg-3′

The given sequence coordinates refer to the published sequences (GenBank accession numbers: Ad12 genome: X73487; Ad2 genome: J01917; preinsertion site in BHK21: X87240).

index of the input solution n_D = 1.3993–4 at 25.0 °C. Loading larger amounts of cellular DNA led to distortions of the DNA profiles. The gradients were centrifuged to equilibrium in a VTi90 rotor of the Beckman XL-100K ultracentrifuge at 35,000 rpm for 24 h at 20 °C. Subsequently, 80-µl fractions were collected from the top of centrifuge tubes using a Hitachi DGF-U density gradient fractionator and a peristaltic pump (Gilson).

Analytical buoyant density gradient centrifugation

About 1 µg of genomic DNA and 1–2 µg of bacteriophage 2C DNA used as density marker were centrifuged to equilibrium in a 50Ti rotor of a Beckman Optima XL-A ultracentrifuge. The sample buffer contained 5 mM NaCl, 10 mM Tris-HCl, pH 7.6, and was adjusted with CsCl to a density of 1.728 g/cm^3. Scans of the gradients were obtained after 24–26 h at 44,000 rpm and were analyzed with appropriate software (Beckman XL-A).

Analyses of the fractionated DNA

The fractionated DNA was analyzed either by dot-blot hybridization or by the polymerase chain reaction (PCR).

For dot-blot analysis, equal portions of each fraction were diluted with 0.4 N NaOH and dot-blotted onto a positively charged nylon membrane (NEN). Subsequently, the DNA on these membranes was hybridized to ^{32}P- or ^{33}P-labeled probes. Autoradiograms were evaluated quantitatively using a Fuji X-BAS 1000 Phosphorimager.

For PCR analyses, aliquots of each fraction were precipitated with ethanol. The pellets were washed with 70% ethanol and resuspended in TE. Portions of these DNA samples were then analyzed by PCR in a Perkin Elmer PE 460 cycler. DNA samples were incubated with appropriate primers (primer sequences and locations are given in table 2), with 0.2 mM of each of the four dNTPs, 3.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 7.0, 0.1% Triton X-100 (w/v) and 2 units Taq polymerase (Sigma). PCR conditions for 10–20 cycles were: 94 °C for 5′, 94 °C for 1′, 68 °C for 45″, 72 °C for 40″, 94 °C for 1′, 62 °C for 45″, 72 °C for 40″. After PCR, portions of each amplicon were loaded on a 2% agarose gel. After electrophoresis, the DNA was blotted onto a positively charged nylon membrane (NEN). Routine procedures for DNA-DNA hybridization and autoradiography were employed. Signal intensities were analyzed with a Phosphorimager.

Results and discussion

The buoyant density profiles of BHK21 hamster DNA and of Ad2 and Ad12 DNAs

The CsCl buoyant density profiles of rodent DNAs exhibit characteristic differences between murids/cricetids and other rodents and can be used to explore rodent systematics [24]. As expected from previous work [14, 24], the CsCl profile of the DNA of the BHK21 hamster cell line derived from *Mesocricetus auratus* was much less heterogeneous than human DNA and did not reveal apparent satellites.

The positions of the profiles of Ad2 and Ad12 DNAs relative to that of hamster DNA were determined in the analytical ultracentrifuge (fig. 1a, b), and the modal GC% levels of Ad2 and Ad12 DNAs were calculated to be 54.6% and 46.1%, respectively. The actual GC% values derived from the published nucleotide sequences were 55.2% [28] and 46.5% [29], respectively.

Mixtures of appropriate amounts of BHK21 DNA and Ad2 or Ad12 DNA were also separated by preparative buoyant density gradient centrifugations (fig. 1c, d). The results were similar to the analytical scans and indicated good separations of BHK21 DNA from Ad2 or Ad12 DNA.

Compositional fractionation of the DNA from Ad12- or Ad2-transformed cell lines or from Ad12-induced tumor cell lines

The DNAs of several Ad2- or Ad12-transformed cell lines (table 1) were fractionated by ultracentrifugation in preparative CsCl buoyant density gradients to determine

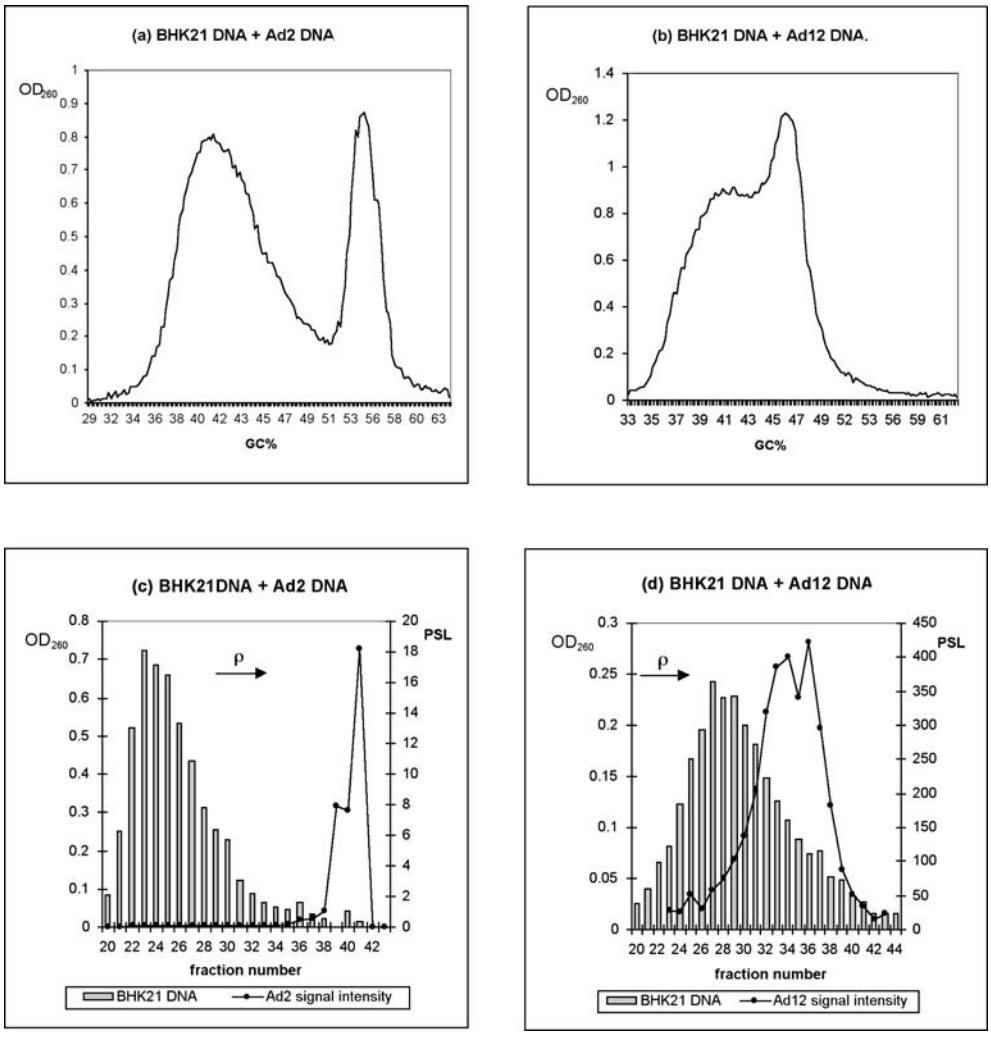

Figure 1. Analytical (*a, b*) and preparative (*c, d*) buoyant density centrifugation of BHK21 DNA mixed with Ad2 DNA (*a, c*) or with Ad12 DNA (*b, d*). Analytical density gradient centrifugation was performed as described in Materials and methods. Phage 2C DNA (ρ_m = 1.742 g/cm^3 [25]) was used as density marker. The formula GC% = (ρ –1.66)/0.00098 [26] was used to calculate the corresponding GC% values. In the preparative buoyant density gradient centrifugation, BHK21 DNA was mixed with eight genome equivalents of Ad2 DNA (*c*), or with eight genome equivalents of Ad12 DNA (*d*) [27]. Centrifugation was performed as described in Materials and methods. Total Ad2 or Ad12 DNA was used as probe in the dot-blot experiments. The adenoviral signal intensity was measured in background-corrected photostimulated luminescence (PSL) units in a Fuji X-BAS 1000 Phosphorimager. Buoyant density ρ increases from the left to the right in Figures 1–3.

the isochores carrying the integrated viral DNAs. Subsequently, gradient fractions were analyzed by dot-blotting on nylon membranes followed by hybridization to probes of Ad2 or Ad12 DNA or by PCR analyses using adenovirus-specific primers.

Figure 2 presents the results obtained with the DNAs from cell line T637 (fig. 2a) and its revertants TR12 (fig. 2c, d) and TR2 (fig. 2b). The Ad12-transformed cell line T637 carries about 15 copies, i.e., approximately 500 kb, of Ad12 DNA, at a single chromosomal locus [30]. In the revertants, Ad12 DNA sequences have been lost. Cell line TR12 contains only 1 copy and a fraction of a second one, TR2 less than 1 copy of Ad12 DNA. However, the chromosomal sites of Ad12 DNA integration are most likely identical (as judged from the similarity of hamster flanking sequences) in cell lines T637, TR12 and TR2 [21; I. Muiznieks and W. Doerfler, unpublished data].

Ad12 DNA could be detected by PCR in a broad range of DNA fractions from cell line T637 using primer pairs from the left terminus of the Ad12 genome. When the Ad12 DNA buoyant density profile in T637 DNA (fig. 2a) was compared to that in the Ad12 DNA + BHK21 DNA reconstitution experiment (fig. 1d), the Ad12 signals in T637 DNA were shifted to DNA fractions of lower GC contents, tailing, however, toward higher values, an indication that mixed cell/viral and pure viral sequences had been released from the very long tandem-repeat inser-

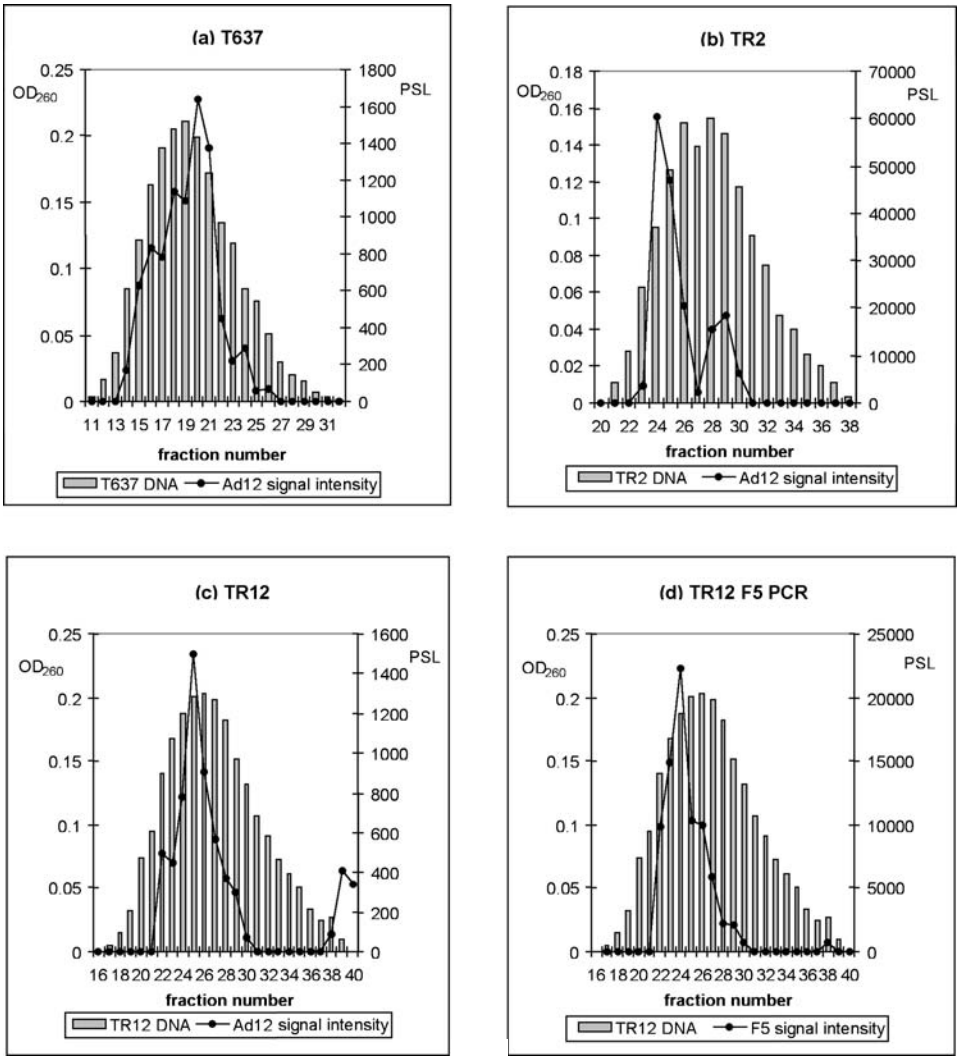

Figure 2. Preparative buoyant density gradient centrifugation of genomic DNA of the Ad12-transformed cell line T637 (*a*) and its revertants TR12 (*c, d*) and TR2 (*b*). Adenoviral sequences were detected by PCR using adenoviral-specific primer pairs L1/L2 or L3/L4 (*a–c*) or primer pair F5-1/F5-2 (*d*) located within the corresponding preinsertion site cloned from the hamster cell line BHK21.

tion. The Ad12 DNA sequences in DNA from cell line T637 showed a rather wide density distribution (fig. 2a). Although the 15 copies of Ad12 DNA are integrated at a single chromosomal location [30], the individual copies are interspersed by cellular and/or rearranged viral DNA [1, 31] and hence give rise to DNA segments of variable GC content and thus a broader density distribution.

The PCR analyses on fractionated DNA from the revertant lines TR12 (fig. 2c, d) and TR2 (fig. 2b) yielded different results in that viral sequences were centered at lighter densities compared to T637 and were different from each other. Finally, the target site for Ad12 DNA integration had a lower GC content than Ad12 DNA, since the target site fragment F5 [21] had its isochore position to the left of the Ad12 DNA signal (fig. 2d). The cloned target site of Ad12 DNA integration in T637 hamster DNA with a length of 5251 bp had a GC content of 36.4% [21].

The Ad12-transformed hamster cell line HA12/7 was derived from primary hamster embryo cells [16]. Each cell carried about three Ad12 DNA equivalents [31]. As for the DNAs from cell lines T637, TR2, and TR12, the data presented in figure 3b again revealed a shift in the GC profile of integrated Ad12 DNA to a lower density, compared to that of free Ad12 DNA (fig. 1d).

After repeated cycles of freezing and thawing of the Ad12-induced hamster tumor cell line CLAC1 [31], the Ad12 DNA was frequently lost from the cells. Traces of Ad12 DNA could, however, be detected by PCR [32] in DNA from the CLAC1 revertant cell line. This finding was confirmed by PCR when primer pairs from the E4 region of Ad12 DNA were used to analyze fractions of a CLAC1 DNA gradient. Amplification products could be detected in a limited number of fractions when 30 PCR cycles were done (fig. 3a). These fractions were close to the

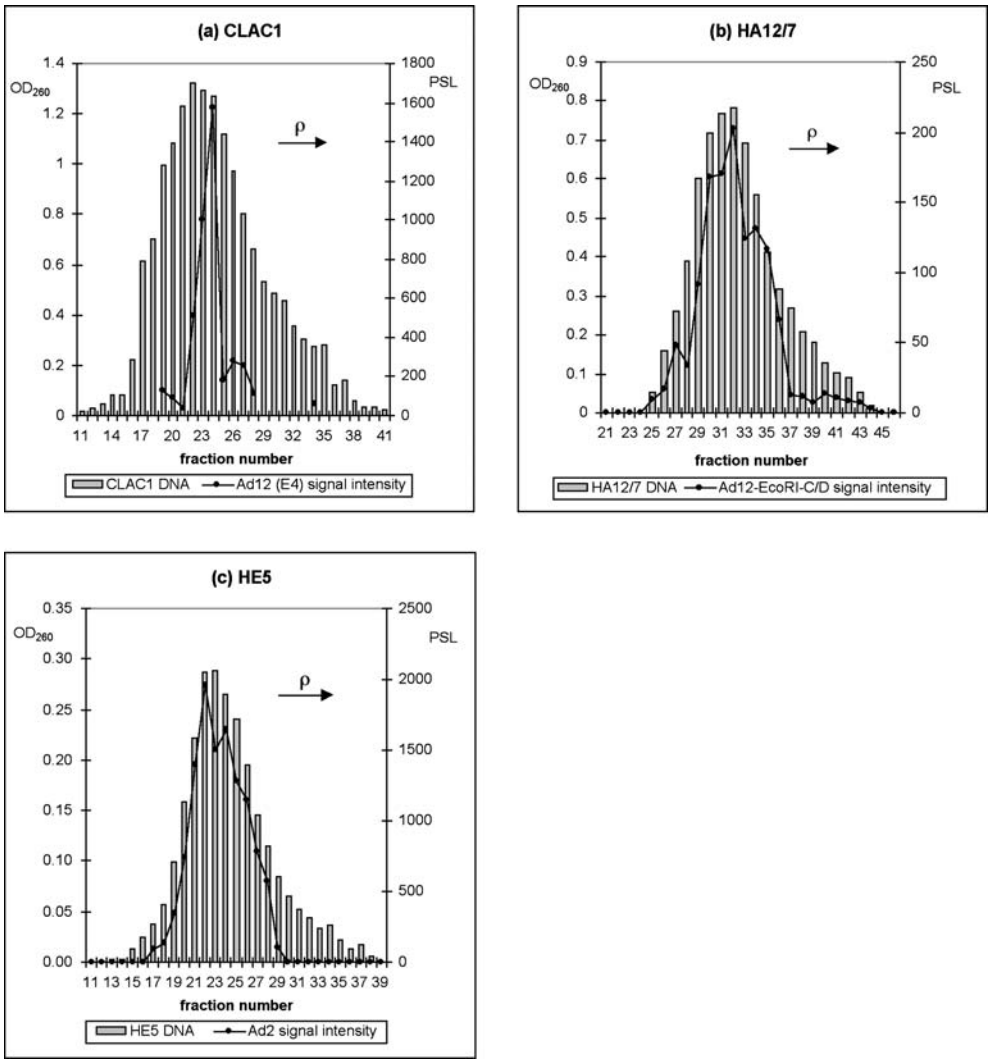

Figure 3. Preparative buoyant density gradient centrifugation of the genomic DNA prepared from the Ad12-induced tumor cell line CLAC1 (*a*), the Ad12-transformed cell line HA12/7 (*b*), or the Ad2-transformed hamster cell line HE5 (*c*). (*a*) PCR with a primer pair specific for the Ad12-E4 region (E4-1/2) was used to amplify adenovirus-specific sequences. (*b*) Equal portions of each fraction were dot-blotted onto a nylon membrane and subsequently hybridized to the cloned *Eco*RI-C and *Eco*RI-D fragments of Ad12 DNA. The signal intensity curve shown represents the mean of two independent dot-blot experiments. (*c*) In the fractions of the HE5 gradient, Ad2 DNA-specific sequences were detected with PCR using Ad2-specific primer pairs (ad2-1/2). The graph summarizes two independent PCR reactions.

isopycnic position of Ad12 DNA (see fig. 1d). Almost the same pattern was found when primer pairs from the E1 region of the Ad12 DNA were used (data not shown).

The Ad2-transformed cell line HE5, established after infecting primary hamster cells with UV-inactivated virions [18], carries about two to three copies of integrated Ad2 DNA [33]. There is a large deletion in the integrated Ad2 genome in this cell line [33]. With primer pairs derived from the left terminus of the Ad2 genome, Ad2-specific signals could be detected in density-fractionated HE5 DNA by PCR in a position corresponding to a much lower GC content than that of Ad2 DNA (fig. 3c, see also fig. 1c). Hence, the chromosomal site of Ad2 DNA integration in cell line HE5 is not isopycnic to Ad2 DNA with a GC content of 55.2%. The results of the isopycnic evaluation are in agreement with the known GC content of 33.6% in the preinsertion site of the hamster cell line HE5 [34].

Interpretation of data

Analyses of Ad12 DNA integration sites by Southern transfer hybridization and by fluorescence in situ hybridization in more than 100 different Ad12-induced hamster tumors have demonstrated that Ad12 DNA can integrate at many different sites in the hamster genome. The integration sites in all cells of one tumor are identical but differ among different tumors [1, 35, 36].

Adenovirus sequences including revertants with short, conserved host-flanking sequences, were found in different isochores of the hamster genome, a result that

suggests integration at many different sites in the host genome as supported by previously adduced results [1, 21, 35] on integration patterns of adenovirus DNA. However, in adenovirus-transformed cell lines continuously cultured for many years, the inserted viral DNA may have been transposed from the original integration site.

Since Ad12 DNA is capable of inserting at multiple sites in the host genome, a mechnanism of insertional mutagenesis appears unlikely to explain adenoviral oncogenesis. One of us (W. D.) is pursuing the possibility that Ad12 DNA integration can lead to global changes in the host genome as already documented by extensive alterations in DNA methylation and transcription patterns in the host genome [30, 36, 37]. These changes may also affect the transcriptional profiles of host genes in Ad12-induced tumor cells [1].

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