JOURNAL OF VIROLOGY, Sept. 1981, p. 684-693
0022-538X/81/090684-10$02.00/0

Vol. 39, No. 3

Arrangement of Integrated Viral DNA Sequences in Cells Transformed by Adenovirus Types 2 and 5

LAMBERTUS VISSER, ANNE-TO D. C. WASSENAAR,
MARIANNE W. VAN MAARSCHALKERWEERD, AND THOMAS H. ROZIJN*

Laboratory for Physiological Chemistry, State University of Utrecht, 3521 GG Utrecht, The Netherlands

Received 29 December 1980/Accepted 20 May 1981

The organization of the viral DNA sequences in 15 adenovirus-transformed cell lines was analyzed by the Southern blotting procedure. The site of adenovirus integration in the cellular genome was found not to be unique, and the viral DNA sequences involved in integration were not confined to a specific region of the adenovirus genome. Several cell lines showed simple integration patterns that demonstrated the presence of large continuous stretches of viral DNA. In four cell lines, containing sequences from both molecular ends of the viral genome, the left- and right-hand-terminal sequences appeared to be linked to each other.

Transformation of rodent cells by adenovirus types 2 and 5 (Ad2 and Ad5) involves integration of viral DNA into the genome of the host cell. The left-hand early region persists in all fully transformed cells. Its expression is necessary for initiation and maintenance of the transformed phenotype (6–8).

Very little is yet known about the recombination processes leading to integration of viral DNA into the cellular genome. In the case of RNA tumor viruses, specific viral sequences appear to be involved in integration, although integrated viral sequences seem to occur at multiple sites in the cellular genome, if not at random (3, 4, 17, 20). No such specificity has been demonstrated for simian virus 40 (2, 10), but more specific patterns of integration have been observed in cells transformed by polyoma virus under special conditions (12).

In cells transformed by adenovirus type 12 (Ad12) or by the Ad5 mutant H5ts125 at the nonpermissive temperature, most or all of the viral genome has been integrated. Moreover, the viral inserts appear to exist in intact linear sequences (5, 19). On the other hand, cells transformed by Ad2 or Ad5 wild-type virus often contain only part of the viral genome (6, 7). It is still a question whether these partial genomes are also present in the form of large integrated sequences colinear with the viral genome or whether these sequences have been fragmented upon integration.

To elucidate the nature of the viral inserts and the possible existence of specific DNA sequences involved in adenovirus integration, we analyzed the organization of the viral DNA sequences in 15 adenovirus-transformed cell lines. The origin and tumorigenicity of these cell lines are listed in Table 1; the DNA content is presented in Fig. 1. All cell lines are T-antigen positive as measured by immunofluorescence (A. Van der Eb, personal communication). Detailed data will be presented elsewhere (M. W. van Maarschalkerweerd, L. Visser, A. C. B. Reemst, A. D. C. Wassenaar, and T. H. Rozijn, manuscript in preparation).

Using the Southern blotting procedure (15) and specific restriction enzyme fragments as labeled probes, we were able to construct integration maps for seven of these cell lines. In all of these cases, one or two large insertions covering the left-hand part of the viral genome were observed. Many other cell lines contained multiple insertions. A close linkage between integrated left-hand-end and right-hand-end viral sequences is suggested for several cell lines.

A preliminary report of some of the data presented here has been published elsewhere (22).

MATERIALS AND METHODS

Cells. The origin of the cell lines studied is described in Fig. 1.

Preparation of cellular and viral DNA. Cellular DNA was isolated either as described by Botchan et al. (2) or by Jeffreys and Flavell (9). In the latter case, treatment with methoxyethanol and further purification were omitted. Both methods result in highly purified high-molecular-weight DNA. The DNA was diluted in 20 mM Tris-hydrochloride (pH 7.5)–0.5 mM EDTA to a concentration of about 1 mg/ml. Ad2 and Ad5 DNAs were purified from virus grown in KB cells as described previously (18, 21).

Restriction endonucleases and cleavage maps. The restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, and *Sma*I were purchased from Boehringer Mannheim Corp. Figure 2 shows the cleavage maps of Ad2 and Ad5.

TABLE 1. *Origin of cell lines*

Line	Cell type	Transforming agent	Tumorigenicity[a]
BHK297-C43	Baby hamster kidney	Fragmented Ad5 DNA	5/5
BHK297-C131	Baby hamster kidney	Fragmented Ad5 DNA	—[b]
BHK268-C31	Baby hamster kidney	Fragmented Ad5 DNA	5/5
BRK424-C1	Rat kidney	Fragmented Ad5 DNA	0/5
5RK-12	Rat kidney	UV-irradiated Ad5 virus	2/5
5RK-15	Rat kidney	UV-irradiated Ad5 virus	0/5
5RK-20	Rat kidney	UV-irradiated Ad5 virus	5/5
RKCVI	Rat kidney	*Hin*dIII-G fragment of Ad5	0/5
5RKRIA	Rat kidney	*Eco*RI-A fragment of Ad5	5/5
RE254-C5	Rat embryo	Ad5 DNA	NT
RE254-2	Rat embryo	Ad5 DNA	5/5
5RE-2	Rat embryo	UV-irradiated Ad5 virus	2/2
ND4-HK-A1678 (ND4)	Hamster kidney	Ad2$^+$ND4	—[c]
A2/HLREF/F1/$T_2C_4(A_2T_2C_4)$	Rat embryo	Ad2 virus	—[d]
Ad2-HK-A2325 (Ad2)	Hamster kidney	Ad2 virus	—[c]

[a] Number of tumors/number of animals. A total of 2×10^6 to 5×10^6 cells were injected subcutaneously into 4-week-old nude mice. Animals were observed weekly for a maximum period of 100 days. The mean latent period was 18 days, except for the cell line 5RK12, where the first visible tumors developed at about 3 months after inoculation. NT, Not tested.

[b] Tumorigenic in nude mice (A. J. van der Eb, personal communication).

[c] Tumorigenic in baby hamsters (S. R. Ross, personal communication).

[d] Tumorigenic in nude mice (P. Gallimore, personal communication).

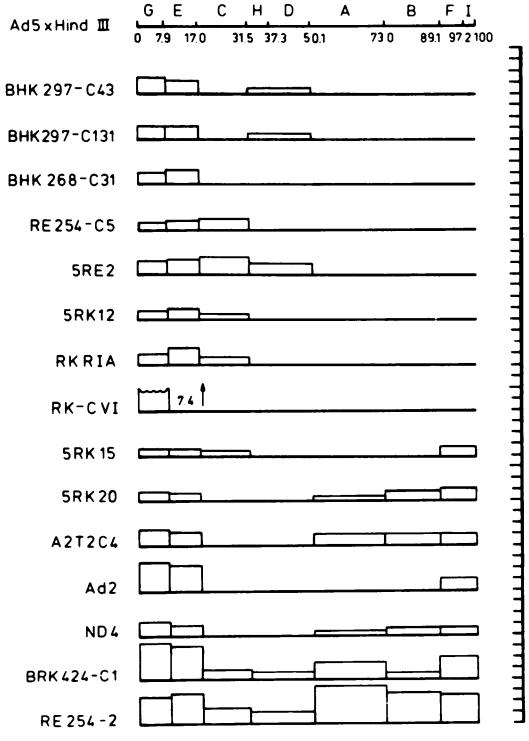

FIG. 1. *Viral DNA content in adenovirus-transformed cell lines. The number of copies of each viral DNA fragment per diploid cell genome equivalent is represented by the height of the blocks. One scale unit corresponds to one copy per diploid cell genome equivalent.*

Isolation of viral restriction fragments. After separation in 0.7% agarose gels, fragments were isolated by electrophoresis into a dialysis bag as described by Steenbergh et al. (16).

Labeling of viral DNA. Radioactive labeling of viral DNA fragments obtained after digestion of DNA with the restriction endonucleases *Hin*dIII, *Eco*RI, and *Sma*I, respectively, was performed by using nick translation by DNA polymerase I (Boehringer Mannheim) as described by Rigby et al. (14), except that digestion with DNase I was omitted. DNAs with a specific activity of more than 2×10^8 cpm/μg were obtained by using [α-^{32}P]dGTP with a specific activity of 2,000 to 3,000 Ci/mmol (The Radiochemical Centre, Amersham). Nonincorporated [α-^{32}P]dTGP was removed by Sephadex G-100 gel filtration followed by ethanol precipitation.

Restriction endonuclease digestion, gel electrophoresis, blotting and filter hybridization of cellular DNAs. Ten micrograms of DNA was digested overnight in 50 μl containing 40 to 50 U of restriction endonuclease, 5 μg of bovine serum albumin (heated at 60°C for 30 min), and the prescribed incubation mix. After addition of 10 μl of bromophenol blue in 50% sucrose, the samples were loaded into slots (10 by 2 by 4 mm) in horizontal 0.7% agarose slab gels (18 by 18 by 0.4 cm; Bio-Rad Laboratories). Gels were run at room temperature at 20 mA, using an electrophoresis buffer containing 40 mM Tris-hydrochloride, 5 mM sodium acetate, and 1 mM EDTA, pH 7.8.

Transfer of DNA to nitrocellulose filters (Sartorius) and filter hybridizations were carried out according to the modification of Jeffreys and Flavell (9) of the Southern technique, including the low-salt wash in 0.015 M NaCl–0.0015 M sodium citrate for 30 min.

Probes were used several times and, before reuse, denatured by adding NaOH to a final concentration of

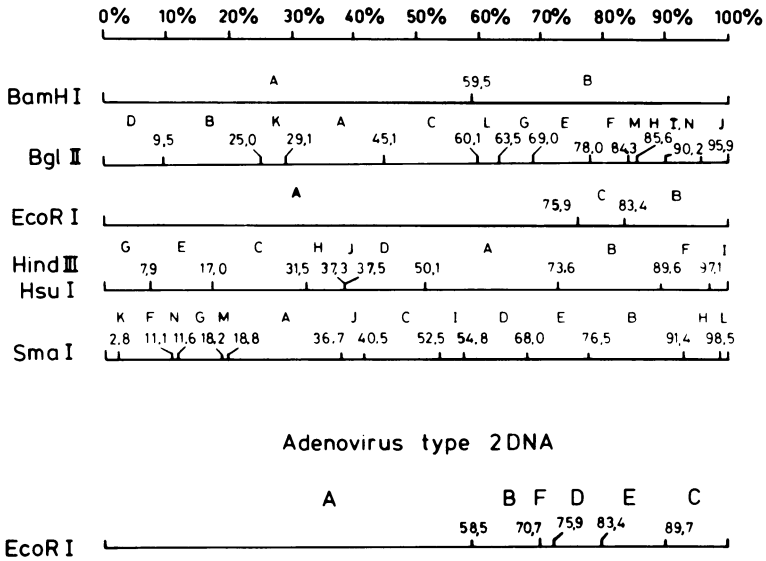

FIG. 2. *Map of the positions at which Ad2 and Ad5 DNA are cleaved by several restriction enzymes. The data were taken from the compilation of V. Mautner (personal communication).*

0.3 N NaOH and neutralization with HCl 10 min later. Filters were autoradiographed by exposure for 3 to 30 days to Fuji-RX medical films used in conjunction with an Ilford intensifying screen at −80°C.

Subcloning of cell lines. Subclones were established by the cloning procedure for anchorage-dependent cells as described by Reid (13). After dilute plating, cells were grown in medium that was conditioned upon half-confluent monolayers of the corresponding cell line for 2 days and filter sterilized afterwards.

RESULTS

To investigate the arrangement of the integrated viral DNA sequences, we digested the DNAs of the transformed cell lines with several restriction endonucleases. The DNA fragments obtained were separated by agarose gel electrophoresis and blotted onto nitrocellulose filters as described by Southern (15). As a reference, we used Ad5 DNA that was similarly fragmented in the presence of calf thymus DNA and coelectrophoresed in each gel. Viral DNA sequences were located on the filters by hybridization with ^{32}P-labeled DNA of the entire viral genome or with viral restriction fragments, followed by autoradiography.

The results allowed us to construct rather simple maps of the integrated viral DNA sequences for seven cell lines, i.e., 5RK12, BHK297-C43, BHK297-C131, BHK268-C31, 5RKIA, 5RE2, and 5RK20. The other cell lines, however, showed more complex integration patterns which could not be interpreted so easily.

Some cell lines contain one or two major viral insertions. (i) 5RK12. The DNA of 5RK12 contained only sequences derived from the left-hand Ad5 *Hin*dIII-G, -E, and -C fragments (Fig. 1). When digested with *Hin*dIII, three fragments appeared that hybridized with ^{32}P-labeled viral DNA (Fig. 3). Fragments 1 and 3 specifically hybridized with the Ad5 restriction enzyme fragment *Hin*dIII-C and the left-hand-terminal fragment *Sma*I-K, respectively, but did not comigrate with the corresponding *Hin*dIII fragments (Fig. 4 and 5). This indicates the presence of cellular sequences joined to the hybridizing viral sequences. Only fragment 2 hybridized with the viral restriction enzyme fragment *Hin*dIII-E and also comigrated with this fragment (Fig. 6), which strongly suggests that this fragment is present in intact form. These data are best explained by assuming the presence of a single contiguous sequence of Ad5 DNA in 5RK12 (see Fig. 8).

(ii) 5RE2. A single insertion of viral DNA was also present in the cell line 5RE2. This cell line contained sequences derived from the left-hand 50% of the Ad5 genome (Fig. 1). Digestion of 5RE2 DNA with *Hin*dIII generated four visible fragments which contained viral DNA (Fig. 3). Fragment 1 hybridized with the Ad5 *Hin*dIII-C fragment and showed comigration in agarose gels with this fragment (Fig. 5). Relatively weak hybridization of this fragment could be observed with a viral *Hin*dIII-E probe due to impurity of this probe (Fig. 6). Fragment 2 specifically hybridized with the viral *Hin*dIII-E fragment and comigrated with this fragment (Fig. 6). Hybridization with the labeled *Sma*I-K probe was detected for fragment 3 (Fig. 4), and this fragment

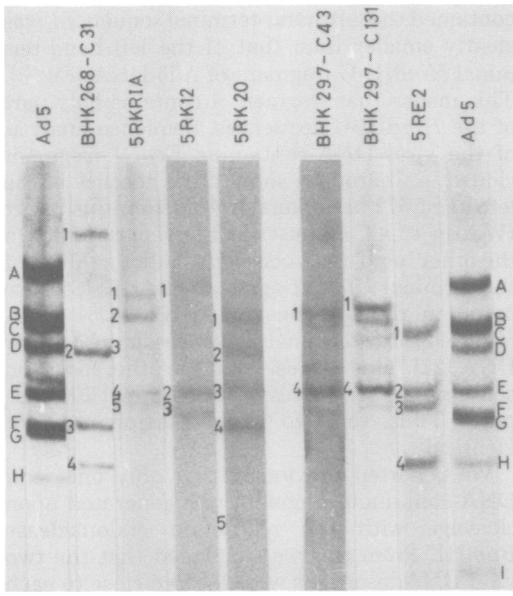

FIG. 3. *Fragment patterns of adenovirus DNA in the genomes of seven cell lines, as revealed after cleavage of the cellular DNA with HindIII restriction endonuclease, blotting, and hybridization with ^{32}P-labeled Ad5 DNA. This figure is a composition of three films. In all gels, bromophenol blue was run approximately the same distance.*

therefore must contain the left-hand-terminal viral sequences. Fragment 4 showed comigration with the Ad5 *Hin*dIII-H fragment and is probably identical to this fragment (Fig. 3). The intact presence of this fragment should result in the generation of a fifth viral DNA-containing fragment which was not reproducibly visible. This can be explained by assuming the presence of only a very short stretch of viral DNA in this fragment. The presence of a single continuous insertion of Ad5 DNA, which is suggested by these data (see Fig. 8), was confirmed by the observation that only one viral DNA-containing fragment was generated by digestion of 5RE2 DNA with *Eco*RI, a restriction endonuclease that has no cleavage site within the left 50% of the viral genome (Fig. 7).

(iii) BHK297-C43 and BHK297-C131. BHK297-C43 and BHK297-C131 contained predominantly viral DNA sequences derived from the left-hand 17% of the viral genome, i.e., the region covered by the Ad5 *Hin*dIII restriction fragments G and E (Fig. 1). Analysis of a *Hin*dIII digest of the DNA of these cell lines by the Southern blotting technique revealed for both lines the presence of two major and some minor fragments that hybridized with a ^{32}P-labeled viral DNA probe (Fig. 3). One of the major fragments (no. 1) hybridized with the *Sma*I-K restriction fragment of Ad5 DNA and therefore contained the left-hand terminal sequences (Fig. 4). The other major fragment (no. 4) hybridized with the viral *Hin*dIII-E restriction fragment and comigrated with this fragment in agarose gels (Fig. 6), indicating the presence of an entire *Hin*dIII-E fragment in these cell lines. One of the minor fragments of each cell line probably contains a DNA sequence derived from a region of the viral genome which is located just to the right of the *Hin*dIII-E fragment. The remaining minor bands represent small insertions which may be derived either from the left-hand end of the viral genome or from the *Hin*dIII-H-D region. Sequences of this region have been detected in these lines by reassociation kinetics analysis (Fig. 1). No clear-cut differences between the two cell lines, generated in one single transfection experiment (F. Graham, personal communication), were observed.

The map constructed for these cell lines is shown in Fig. 8.

(iv) BHK268-C31. The cell line BHK268-C31 resembles to some extent BHK297-C43 and -C131 in that it contains sequences derived from the Ad5 *Hin*dIII-G and -E fragments (Fig. 1).

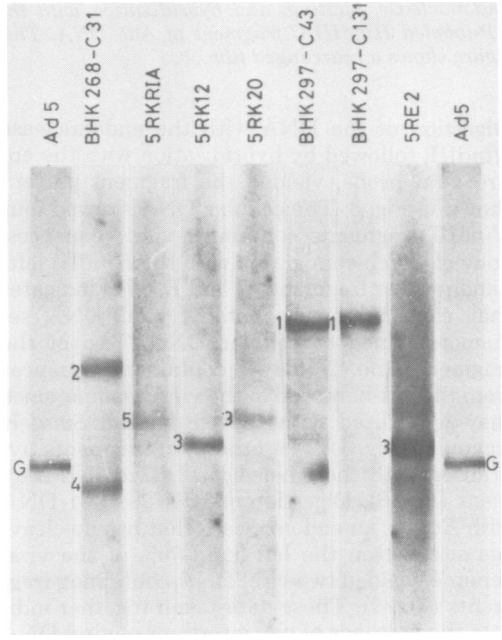

FIG. 4. *Fragment patterns of adenovirus DNA in the genomes of seven cell lines, as revealed after cleavage of the cellular DNA with HindIII restriction endonuclease, blotting, and hybridization with the ^{32}P-labeled SmaI-K fragment of Ad5 DNA. This figure is a composition of two films. In both gels, bromophenol blue was run approximately the same distance.*

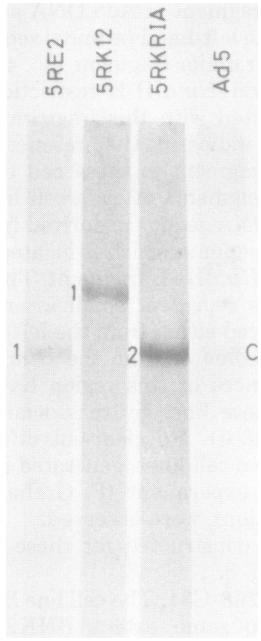

FIG. 5. *Fragment patterns of adenovirus DNA in the genomes of three cell lines, as revealed after cleavage of the cellular DNA with HindIII restriction endonuclease, blotting, and hybridization with the ^{32}P-labeled HindIII-C fragment of Ad5 DNA. This figure shows a rearranged film.*

Digestion of the DNA with the endonuclease *Hin*dIII, followed by hybridization with the entire viral probe, yielded the fragment pattern shown in Fig. 3. The cellular DNA showed four *Hin*dIII fragments containing viral sequences, none of which comigrated with the *Hin*dIII left-hand marker fragments G and E. This indicates that each fragment contains viral DNA sequences joined to cellular DNA. Two of the fragments (no. 2 and 4) contained sequences from the left-hand end of the viral genome, since they hybridized with the ^{32}P-labeled *Sma*I-K fragment (Fig. 4). The other two fragments hybridized with the labeled viral *Hin*dIII-E fragment (Fig. 6). Digestion of BHK268-C31 DNA with *Eco*RI, an endonuclease that has no cleavage site within the left-hand 75% of the viral genome, yielded two viral DNA-containing fragments (Fig. 7). These data taken together indicate the presence of two insertions of viral DNA in this cell line. Restriction enzyme analysis of one of the two viral DNA-containing *Eco*RI fragments, cloned in Charon 4A, showed that the *Hin*dIII fragments 3 and 4 are located in the same *Eco*RI fragment (Westin et al., manuscript in preparation).

The mass of the *Hin*dIII fragment 4, which contained the left-hand-terminal sequences, was clearly smaller than that of the left-hand-terminal *Hin*dIII-G fragment of Ad5 DNA (Fig. 3). This means that fragment 4 contains only part of the *Hin*dIII-G sequences. Sequence analysis of the viral DNA-containing *Eco*RI fragment cloned in Charon 4A showed the absence of the left-hand 572 base pairs of Ad5 from this insert (Westin et al., manuscript in preparation). On the other hand, reassociation kinetics analysis of the terminal *Hpa*II fragments of Ad5 (188 base pairs) in the presence of BHK268-C31 DNA revealed these terminal sequences in the cellular DNA (22). This suggests that the other insertion contains the sequences of the terminal 200 base pairs. Thus, the two viral insertions are not identical.

We reported previously that only one viral DNA-containing fragment was generated upon cleavage with the restriction endonuclease *Bam*HI. From this we concluded that the two viral DNA insertions were located close to each other (22). However, a more careful analysis

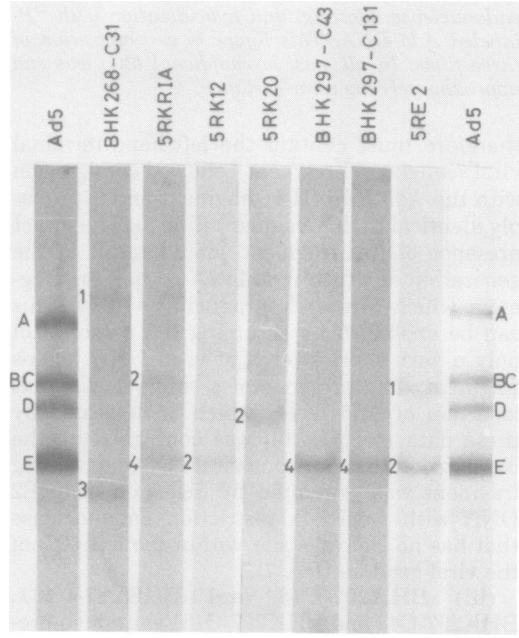

FIG. 6. *Fragment patterns of adenovirus DNA in the genomes of seven cell lines, as revealed after cleavage of the cellular DNA with HindIII restriction endonuclease, blotting, and hybridization with the ^{32}P-labeled HindIII-E fragment of Ad5 DNA. This figure is a composition of two films. In both gels, bromophenol blue was run approximately the same distance. The two lanes containing Ad5 DNA show contamination of the ^{32}P-labeled HindIII-E fragment with the larger HindIII fragments.*

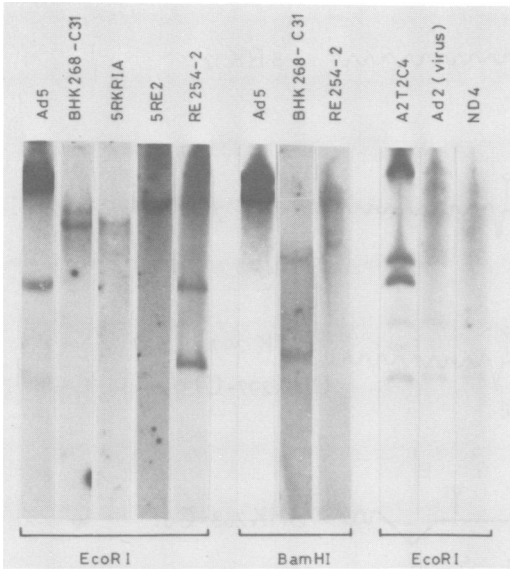

FIG. 7. *Fragment patterns of adenovirus DNA in the genomes of three cell lines, as revealed after cleavage of the cellular DNA with EcoRI or BamHI restriction endonuclease, blotting, and hybridization with ^{32}P-labeled Ad5 DNA. This figure is a composition of three films. In all gels, bromophenol blue was run approximately the same distance, except for the BamHI digest of BHK268-C31, which gel was run three times as long.*

revealed the presence of an additional *Bam*HI fragment containing viral sequences (Fig. 7), which had been overlooked in previous studies. Its large size might have been responsible for a poor transfer from the agarose gel to the nitrocellulose filter. This would explain the weak hybridization with Ad5 DNA obtained for this fragment. This finding leaves only the possibility of two viral insertions, present in the DNA of this cell line, which are not closely linked and rules out the model we have proposed previously (22). A new model is depicted in Fig. 8. Double digests of cellular DNA with *Bam*HI, *Hin*dIII, and *Eco*RI and data obtained from analysis of the *Eco*RI fragment, cloned in Charon 4A, confirmed this model (data not shown).

(v) 5RKRIA. DNA of the cell line 5RKRIA contained sequences of the left-hand 32% of the viral genome (Fig. 1). Upon digestion with the restriction endonuclease *Hin*dIII, 5RKRIA DNA yielded five fragments hybridizing to labeled viral DNA of the entire genome (Fig. 3). Fragments 2, 4, and 5 hybridized with the Ad5 *Hin*dIII-C, *Hin*dIII-E, and *Sma*I-K fragments, respectively (Fig. 4–6). Moreover, fragments 2 and 4 comigrated with the corresponding viral fragments. This suggested the presence of a major contiguous viral insertion, ranging from the left-hand end past the *Hin*dIII cleavage site at 32% of the Ad5 map. Digestion with the restriction endonuclease *Eco*RI generated two major fragments hybridizing with Ad5 DNA (Fig. 7). Since no *Eco*RI recognition site was present in the viral sequences detected in this cell line, this shows the presence of an additional viral insert. The two remaining *Hin*dIII fragments, 1 and 3, probably represent sequences originating from this second viral insert.

The cleavage map is shown in Fig. 8.

(vi) 5RK20. The viral sequences integrated in 5RK20 were organized in a peculiar way. This cell line contained sequences derived from both left- and right-hand-terminal regions of the viral genome (Fig. 1). Cleavage of 5RK20 DNA with *Hin*dIII generated five visible fragments containing viral DNA (Fig. 3). Fragments 1 and 4 specifically hybridized with the right-hand Ad5 *Eco*RI-B restriction fragment and comigrated with the viral *Hin*dIII fragments B and F, respectively, which share sequences with the Ad5 *Eco*RI-B fragment (Fig. 9). Fragment 2 hybridized with the viral *Hin*dIII-E fragment but did not comigrate with this fragment (Fig. 6).

Fragment 3 hybridized with both the ^{32}P-labeled *Eco*RI-B fragment and the *Sma*I-L fragment of Ad5 DNA, the latter covering the right-hand 2% of the viral genome (Fig. 9). This fragment should therefore contain Ad5 *Hin*dIII-I sequences. However, fragment 3 was also the only fragment that hybridized with the ^{32}P-labeled Ad5 *Sma*I-K probe (Fig. 9). A similar fragment, likewise hybridizing with both the *Sma*I-K and -L probes, was generated upon cleavage of 5RK20 DNA with *Bgl*II (data not shown). This observation cannot be ascribed to cross-hybridization due to the inverted terminal repetitions (15), since in the control digests containing Ad5 DNA only the *Hin*dIII-G and -I fragments became visible upon hybridization with the ^{32}P-labeled *Sma*I-K and -L probes, respectively (Fig. 9). Rather, it is likely that in this cell line the integrated sequences derived from both the left- and right-hand viral genome termini are closely linked, as depicted in Fig. 8. Fragment 3 was just about the size of the Ad5 *Hin*dIII-G and -I fragments together. This means that the terminal viral sequences integrated in this cell line are located very close to each other, possibly separated by only a short stretch of cellular DNA. That the integrated viral sequences are not contiguous but indeed separated from each other by cellular DNA sequences could be deduced from the presence of an *Eco*RI cleavage site in between them (data not shown). Similar observations were made for some other cell lines as discussed below.

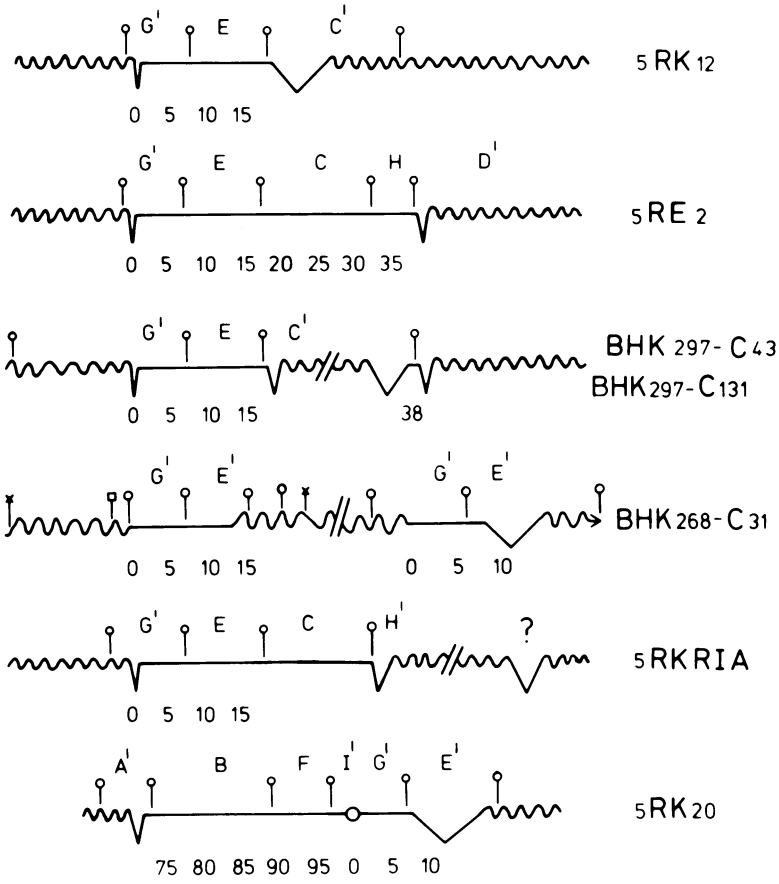

FIG. 8. *Restriction enzyme maps of Ad5 DNA insertions present in the DNA of seven transformed cell lines. The maps were constructed by using the data shown in the preceding figures. Symbols: (⚲) a cleavage site of HindIII; (⚸) cleavage site of EcoRI; (⚳) cleavage site of BamHI. The solid lines represent integrated Ad5 DNA; the wavy lines represent flanking cellular DNA sequences. The dips at the junctions of viral and cellular DNA represent segments of unknown origin. The length of these segments is not known accurately.*

Multiple or rearranged DNA insertions corresponding to various segments of the viral genome are present in several cell lines. *Hin*dIII digestion of the DNAs of the cell lines BRK424-C1, RE254-2, 5RK15, RE254-C5, $A_2T_2C_4$, Ad2, and ND4, followed by hybridization to a labeled probe of the entire viral genome, yielded the fragment patterns shown in Fig. 10. Some of the viral sequences containing *Hin*dIII fragments, observed in the DNA of cell lines BRK424-C1, RE254-2, $A_2T_2C_4$, Ad2, and ND4, hybridized neither to the ^{32}P-labeled *Hin*dIII-E fragment (Fig. 10) nor to the *Sma*I-K fragment (not shown). These fragments therefore contained viral sequences that were not derived from the left-hand-terminal region.

In general, the virus-specific fragments observed did not comigrate with the Ad5 DNA marker fragments. This indicates that in these cell lines most of the integrated viral sequences are arranged in a fashion not colinear with the Ad5 genome DNA. An exception was formed by the comigration frequently observed for the viral *Hin*dIII-E fragment covering the nonterminal part of early region 1. In four cases, i.e., for the cell lines BRK424-C1, RE254-2, 5RK15, and Ad2, comigration of fragments that specifically hybridized with the viral *Hin*dIII-E fragment was observed, which suggests the intact presence of this DNA fragment (Fig. 10).

*Eco*RI digestion of DNA of the cell lines $A_2T_2C_4$ and ND4 generated virus-specific fragments which comigrated with the viral Ad2 *Eco*RI-D and -F fragments in the case of $A_2T_2C_4$ and with the *Eco*RI-F fragment in the case of ND4 (Fig. 7). This finding suggests the presence of larger intact sequences from the right-hand part of the viral genome in these cell lines.

Whereas digestion of RE254-2 DNA with *Hin*dIII endonuclease generated 12 virus-spe-

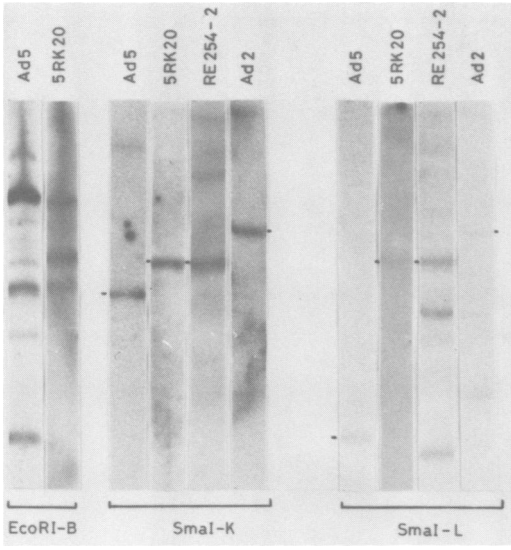

FIG. 9. *Fragment patterns of adenovirus DNA in the genomes of three cell lines, as revealed after cleavage of the cellular DNA with HindIII restriction endonuclease, blotting, and hybridization with the ^{32}P-labeled EcoRI-B, SmaI-K, or SmaI-L fragment of Ad5 DNA. This figure is a composition of three films. In all gels, bromophenol blue was run approximately the same distance.*

cific fragments, most of which did not comigrate with *Hin*dIII marker fragments, a smaller number of fragments was obtained by *Eco*RI and *Bam*HI digestion (Fig. 7). This indicates either that in this cell line viral DNA has been integrated in the cellular genome at multiple sites, which are, however, confined to a few narrow regions of the cellular genome, or that extensive rearrangements of viral sequences have taken place after an original integration of one or more large stretches of viral DNA.

Digestion of the DNA of the *Hin*dIII-G-transformed cell line 5RKCVI with the endonucleases *Hin*dIII, *Eco*RI, and *Bam*HI led to the appearance of eight, six, and four virus-specific fragments, respectively (data not shown). Since none of these enzymes recognizes the viral sequences present in this cell line, in this case a clustering of the viral sequences best explains this finding.

In some cell lines integrated viral termini are linked. A close linkage of integrated right- and left-hand viral DNA sequences, similar to that observed in 5RK20 DNA, was also suggested by the hybridization data obtained for the cell lines RE254-2 and Ad2. One *Hin*dIII fragment of the DNA of these cell lines hybridized with both the *Sma*I-K and -L probes (Fig. 9). In the case of RE254-2, the size of these fragments was approximately the sum of the two terminal *Hin*dIII fragments of Ad5 DNA. A similar fragment detected in the cellular Ad2 DNA was larger in size and should therefore contain a larger stretch of cellular sequences. Using restriction enzyme *Bgl*II instead of *Hin*dIII, this linkage phenomenon was confirmed (data not shown). Preliminary experiments also suggested this linkage for the cell line $A_2T_2C_4$.

Stability and homogeneity of viral DNA sequences in transformed cells after prolonged subcultivation and subcloning. To investigate whether the viral DNA sequences remained stably integrated during subcultivation, we cloned cells of the cell lines BRK424-C1, RE254-2, 5RK12, 5RE2, and 5RK20 after prolonged subculturing. Four subclones of each cell line were grown. DNA of each subclone was digested with *Hin*dIII, blotted, and hybridized to labeled viral DNA. Except for BRK424-C1, fragment patterns detected were identical to those obtained with the DNA of the parental lines.

The cell line BRK424-C1 exhibited a less stable viral integration pattern. Reassociation kinetics analysis on DNA isolated after approximately 100 passages in vitro revealed the loss of most of the viral DNA sequences from the middle region of the viral genome between map positions 20 and 70. Coinciding with this loss of integrated viral DNA sequences, cells with a less transformed, more fibroblastic morphology made their appearance in the population BRK424-C1 cells. Clones of these apparently less transformed cells contained no viral DNA as determined by blotting experiments and by reassociation kinetics analysis.

DISCUSSION

In our cell lines, the viral DNA is present in relatively low amounts, ranging from one to four copies of the various viral DNA segments per diploid quantity of transformed cell DNA (Fig. 1). These cell lines are therefore very suitable for studying the arrangement of the integrated viral DNA sequences. For 7 of 15 cell lines, we were able to construct maps of the integrated viral sequences.

The data obtained with the Southern blotting technique clearly indicate that the arrangement of the integrated adenovirus DNA sequences is quite different in independently isolated clones of transformed cell lines. No correlation between integration pattern and either cell type, transforming agent, or tumorigenicity could be deduced.

The *Hin*dIII fragments of transformed cell DNA that contain left-hand-end viral sequences

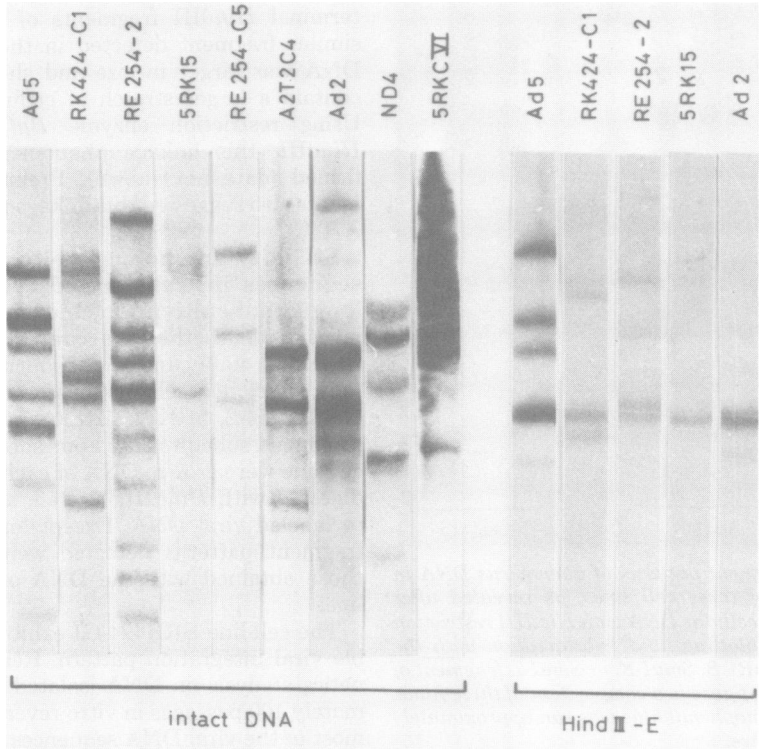

FIG. 10. *Integration patterns of adenovirus DNA in the genomes of eight cell lines, as revealed after cleavage of the cellular DNA with HindIII restriction endonuclease, blotting, and hybridization with either ^{32}P-labeled Ad5 DNA or the ^{32}P-labeled HindIII-E fragment of Ad5 DNA. This figure is a composition of four films. In all gels, bromophenol blue was run approximately the same distance.*

joined to cellular DNA sequences vary in length in the different cell lines. This indicates that the cellular integration site is not identical in the different transformed cell lines. Similar observations are reported by Sutter et al. (19) for Ad12-transformed cell lines, by Dorsch-Häsler et al. (5) for cell lines transformed by Ad5 ts125 at the nonpermissive temperature, and by J. Sambrook and by L. Vardimon and W. Doerfler for Ad2-transformed cell lines (both personal communication).

Five cell lines, BHK297-C43, BHK297-C131, 5RK12, 5RE2, and 5RKRIA, apparently contain one major insertion, consisting of a contiguous stretch of viral DNA, ranging from the left-hand viral genome terminus to map positions between 15 and 50 map units. A sixth cell line, BHK268-C31, contains two such insertions, which are not identical. In several other cell lines, no such colinearity of larger stretches of viral DNA appears to exist, as judged from the absence of comigration with viral marker fragments in *Hin*dIII digests of DNAs of these cell lines.

The absence of comigration observed to some extent in the cell lines Ad2 and T_2C_4, which are transformed by virus, may be explained in two ways. This finding may reflect the presence either of large but noncolinear viral insertions resulting from secondary rearrangements of the integrated sequences or of multiple independent insertions of viral sequences of random segments of the viral genome. A similar explanation can be given for the observed fragment patterns in cell lines generated by transfection with intact viral DNA (e.g., RE254-2).

If multiple independent viral insertions are present in the genome of RE254-2, our data suggest that these insertions are clustered. Such a clustering is also suggested for the cell line 5RKCVI, which was transfected by the Ad5 *Hin*dIII-G fragment. In this way, the arrangement of the viral sequences in these cell lines might be similar to the arrangement of exogenous DNA sequences in the cells described by Perucho et al. (11), which were also transfected by DNA. They demonstrated that exogenously acquired sequences can be genetically linked and that their flanking sequences derive primarily from the carrier species rather than the host species.

Our studies bear only on the final arrangement of the viral insertions in the transformed cell

genome. Hence, it is difficult to say whether multiple independent insertions or secondary rearrangements within the integrated viral sequences have caused the absence of comigration observed in the above-mentioned cell lines. As a result, it remains unclear whether the left-hand early region which encompasses the transforming genes or the molecular ends of the viral genome as the origins of replication are necessarily or preferentially involved in some step of the integration process.

Four cell lines, 5RK20, RE254-2, Ad2, and $A_2T_2C_4$, exhibit a peculiar integration pattern. In these cell lines, which contain DNA sequences derived from both the left- and right-hand-terminal region of the viral genome, these sequences appear to be linked to each other, possibly separated by a short stretch of cellular DNA. Similar findings are reported by Sambrook and by Vardimon and Doerfler (personal communication). Such an arrangement might be purely accidental, being the product of two independent insertions very close to each other. But the occurrence in 4 of 15 transformed cell lines makes it tempting to think of an integration event in which a circular form of adenovirus DNA, perhaps stabilized by the terminal proteins, is involved.

Taken together, our data on the arrangement of integrated viral DNA sequences imply that the site of adenovirus integration in the cellular genome is not unique and that the viral DNA sequences present are not confined to a specific region of the adenovirus genome. Moreover, most of these integration patterns are stable and do not change during subcultivation as judged by subcloning. However, one of the cell lines shows partial to total loss of the integrated viral sequences.

At present, we are using recombinant DNA techniques to clone the integrated viral DNA and its flanking cellular sequences from several transformed cell lines. We will use these clones to determine the sequences that join the viral to the cellular DNA and to study the molecular organization of the integrated viral sequences in more detail.

ACKNOWLEDGMENT

This investigation was supported in part by the Foundation for Medical Research FUNGO, which is subsidized by the Netherlands Organization for Advancement of Pure Research.

LITERATURE CITED

1. **Botchan, M., G. McKenna, and P. A. Sharp.** 1973. Cleavage of mouse DNA by a restriction enzyme as a clue to the arrangement of genes. Cold Spring Harbor Symp. Quant. Biol. **38**:383–395.
2. **Botchan, M., W. Topp, and J. Sambrook.** 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell **9**:269–287.
3. **Cohen, J. C., P. R. Shank, V. L. Morris, R. Cardiff, and H. E. Varmus.** 1979. Integration of the DNA of mouse mammary tumor virus in virus-infected normal and neoplastic tissue of the mouse. Cell **16**:333–345.
4. **Collins, C. J., and J. T. Parsons.** 1977. Integration of avian sarcoma virus DNA sequences in transformed mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **74**:4301–4305.
5. **Dorsch-Häsler, K., P. B. Fisher, I. B. Weinstein, and H. S. Ginsberg.** 1980. Patterns of viral DNA integration in cells transformed by wild type or DNA-binding protein mutants of adenovirus type 5 and effect of chemical carcinogens on integration. J. Virol. **34**:305–314.
6. **Gallimore, P. H., P. A. Sharp, and J. Sambrook.** 1974. Viral DNA in transformed cells. J. Mol. Biol. **89**:49–72.
7. **Graham, F. L., P. J. Abrahams, C. Mulder, H. L. Heyneker, S. O. Warnaar, F. A. J. de Vries, W. Fiers, and A. J. van der Eb.** 1974. Studies on in vitro transformation by DNA and DNA fragments of human adenoviruses and simian virus 40. Cold Spring Harbor Symp. Quant. Biol. **39**:637–650.
8. **Graham, F. L., T. Harrison, and J. Williams.** 1978. Defective transforming capacity of adenovirus type 5 host-range mutants. Virology **86**:10–21.
9. **Jeffreys, A. J., and R. A. Flavell.** 1977. A physical map of the DNA regions flanking the rabbit β-globin gene. Cell **12**:429–440.
10. **Ketner, G., and T. J. Kelly, Jr.** 1976. Integrated simian virus 40 sequences in transformed cell DNA: analysis using restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. **73**:1102–1106.
11. **Perucho, M., D. Hanahan, and M. Wigler.** 1980. Genetic and physical linkage of exogenous sequences in transformed cells. Cell **22**:309–317.
12. **Rassoulzadegan, M., E. Mougneau, B. Perbal, P. Gaudray, F. Birg, and F. Cuzin.** 1979. Host-virus interactions critical for cellular transformations by polyoma virus and SV40. Cold Spring Harbor Symp. Quant. Biol. **44**:333–342.
13. **Reid, L. C. M.** 1979. Cloning of anchorage-dependent cells. Methods Enzymol. **58**:158–159.
14. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
15. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
16. **Steenbergh, P. H., J. Maat, H. van Ormondt, and J. S. Sussenbach.** 1977. The nucleotide sequence at the termini of adenovirus type 5 DNA. Nucleic Acids Res. **4**:4371–4389.
17. **Steffen, D., and R. A. Weinberg.** 1978. The integrated genome of murine leukemia virus. Cell **15**:1003–1010.
18. **Sussenbach, J. S., and P. C. van der Vliet.** 1973. Studies on the mechanism of replication of adenovirus DNA. I. The effect of hydroxyurea. Virology **54**:299–303.
19. **Sutter, D., M. Westphal, and W. Doerfler.** 1978. Patterns of integration of viral DNA sequences in the genomes of adenovirus type 12-transformed hamster cells. Cell **14**:569–585.
20. **Van der Putten, H., E. Terwindt, and A. Berns.** 1979. The integration sites of endogenous and exogenous Moloney murine leukemia virus. Cell **18**:109–116.
21. **Van der Vliet, P. C., and J. S. Sussenbach.** 1972. The mechanism of adenovirus DNA synthesis in isolated nuclei. Eur. J. Biochem. **30**:484–492.
22. **Visser, L., M. W. van Maarschalkerweerd, T. H. Rozijn, A. D. C. Wassenaar, A. M. C. B. Reemst, and J. S. Sussenbach.** 1979. Viral DNA sequences in adenovirus transformed cells. Cold Spring Harbor Symp. Quant. Biol. **44**:541–550.