Tumor Res. **19**, 49–61 (1984)

Analysis of Viral Sequences Integrated and Expressed in Rat Cell Lines Transformed by the Leftmost 4.7% Fragment of Adenovirus Type 12 DNA

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SUMMARY

We have analyzed the integration and expression of viral sequence in HY cells with incomplete phenotypes which were previously established by transfecting rat cells with the leftmost DNA fragment *Acc*I-H of adenovirus (Ad) type 12. Southern blot analyses showed that the state of integrated viral sequences is nearly identical for the three cell lines isolated from independent transformed foci. Approximately 10 copies of the AccI-H sequence were integrated within a region of 22 kb, mostly separated from each other by various spacer sequences, in a high-molecular-weight DNA from the cell line HY1-5. Most of the integrated viral DNA copies carried whole transcription unit of the ElA gene. Sl mapping experiments revealed the presence of the ElA mRNA species of normal structure in these cells. These results suggested that HY cells can synthesize genuine ElA polypeptides and that the incompleteness of their phenotypes is not due to the absence or alteration of some of the entire genetic informations of the ElA region.

Key words: Adl2 ElA, Incomplete transformation, Integration, Southern blot analysis, Sl analysis

INTRODUCTION

All the transformed cells induced by human adenoviruses carry viral sequences integrated into the cellular DNA. The viral sequences are transmitted to progeny cells as genetic elements indistinguishable from other cellular genes. Analyses on adenoviral transformants have revealed no specific integration sites either in cellular or viral sequence (5, 7, 13, 17). The only common feature is the integration and expression of El genes in all transformed cells so far analyzed. It has been confirmed by transfecting subgenomic DNA fragments that the adenoviral

El genes are sufficient to induce cell transformation (8, 18, 19, 26).

Further studies with DNA transfection have demonstrated that the ElA gene alone has some ability to induce transformation of cells (4, 20, 23). Since the resulting ElA transformants lack some of the typical phenotypes, it is referred to incomplete or atypical transformation (4, 20, 23). Although these cells have been shown to carry viral sequences transfected, it remains to be elucidated whether or not they carry all the ElA sequence to express the intact ElA gene products.

In this communication, we report the integration and expression of the ElA gene in transformed rat cell lines induced by transfecting the leftmost fragment *Acc*I-H (1597 bp; 4.7%) of adenovirus type 12 DNA. Multiple copies of viral sequence were integrated in a limited region of cellular DNA in nearly the same manner for cell lines established from independent transformed foci. Typical ElA mRNA species were found in all cell lines.

MATERIALS AND METHODS

Cells and virus

HY1-1, HY1-5, HY2-1 and HY7-1 cells were established from three independent transformed foci induced by transfecting a rat cell line 3Y1 with the leftmost DNA fragment *Acc*I-H (1597 nucleotide pairs; 4.7%) of Ad12 DNA (20). HY1-1 and HY1-5 were established from the same original focus by recloning. These cells were cultured in Eagle's MEM containing 0.1 mM $CaCl_2$ and supplemented with 10% fetal bovine serum (GIBCO Laboratory). Adl2 Strain Huie was propagated in human KB cells.

DNA and RNA preparations

High-molecular-weight cellular DNA was prepared according to the method of Blin and Stafford (2) as described by Maniatis *et al.* (12). Cells were suspended in 0.01 M Tris (pH 7.5)-0.001 M EDTA and lysed by adding 10 volumes of a solution of 0.5 M EDTA (pH 8.0), 200 μg proteinase K/m*l* and 0.5% Sarkosyl. After incubation at 37 ℃ overnight, the lysate was extracted gently with phenol three times and dialyzed against 0.01 M Tris (pH 8.0), 0.001 M EDTA and 0.01 M NaCl (TNE) extensively. Samples were treated with 100 μg DNase-free RNase A/m*l* and then with 50 μg proteinase K/m*l*. DNA samples were extracted twice with phenol/chloroform and dialyzed extensively against TNE.

Viral DNA was prepared from purified virions as described by Green and Pina (9). Viral DNA fragments were prepared by cleaving viral DNA with restriction endonucleases followed by agarose gel electrophoresis. Viral DNA fragments were also prepared from recombinant plasmids pAdC22 which carried Ad12 *Eco*RI-C fragment in pBR325 (Sugisaki *et al.*, manuscript in preparation) and from pASC-1

which contains Ad12 *Sal*I–C fragment in pBR322 (6). According to nucleotide sequence data (22), restriction maps of several enzymes within the *Acc*I–H fragment of Ad12 DNA are shown in Fig. 1 together with the structure of ElA mRNA species (15).

The cytoplasmic fraction was prepared by hypotonic lysis with 0.5% NP–40 according to Zieve and Penman (27). It was extracted with phenol/chloroform after an addition of 0.5% sodium dodecyl sulfate (SDS), 0.01 M EDTA and 0.1 M NaCl. RNA was precipitated by adding two volumes of ethanol and applied on a column of oligodeoxythymidilic acid–cellulose to select polyadenylic acid–containing fraction as described by Maniatis *et al.* (12).

Southern blot analysis

High-molecular-weight DNA was cleaved with restriction endonucleases, electrophoresed on 1.0% agarose gels, denatured *in situ*, and transferred to nitrocellulose membrane filters by a modification of the technique of Southern (21)

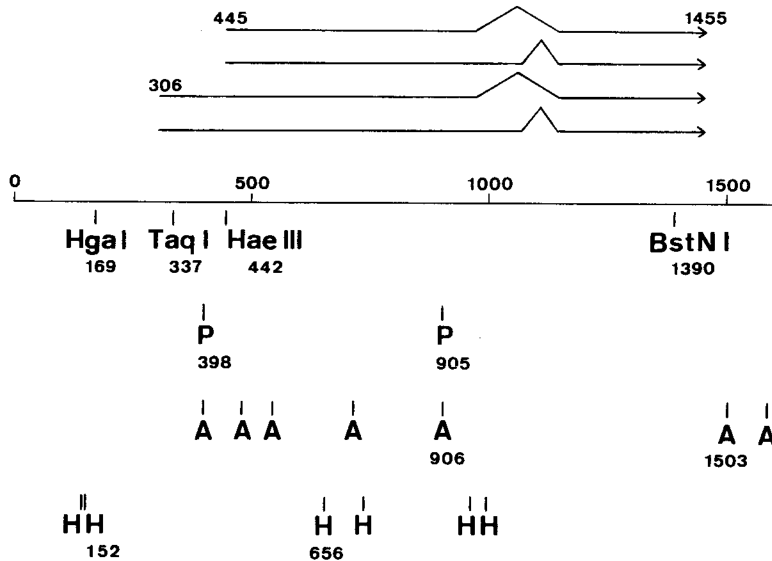

Fig. 1 Configuration of Ad12 ElA gene.
The coordinate represents *Acc*I–H fragment (1597 nucleotides, leftmost 4.7%) of Ad12 DNA which codes for the whole ElA transcription unit. Four mRNA species identified in lytically infected cells (15) are shown by arrows. Numerics show the positions of transcription start and polyadenylation sites in nucleotide numbers from the left end of the genome. Restriction endonucleases *Hga*I, *Taq*I, *Hae*III and *Bst*NI cleave the *Acc*I–H fragment once at the position indicated in the figure. Enzymes which have more than one cleavage sites are also shown: P, *Pvu*II; A, *Alu*I; H, *Hap*II.

as described in *Molecular Cloning* (12). DNA blots were hybridized to Ad12 DNA fragments labeled with [α^{32}P]dATP and [α^{32}P]dCTP by the nick translation reaction (11). Hybridization was carried out at 45 ℃ overnight in a solution containing 0.02 M Tris HCl (pH 7.5), 0.6 M NaCl, 1 mM EDTA, 1×Denhardt solution, 100 μg denatured and sonicated calf thymus DNA/ml, 0.05% SDS and 50% deionized formamide. The filters were washed twice in 2×SSC+0.1% SDS at room temperature, four times in 0.2×SSC+0.1% SDS at 68 ℃ and exposed to X-ray films with intensifying screens.

RNA blot analysis

Polyadenylic acid-containing RNA was treated with glyoxal and dimethylsulfoxide and electrophoresed on a 1.5% agarose gel according to McMaster and Carmichael (13) as described previously (15). RNA was transferred to a nitrocellulose filter at 4 ℃ overnight. RNA blot was baked in a vacuum oven at 80 ℃ for 2 hours and incubated at 45 ℃ overnight in a solution containing 1% glycine and 1 mg denatured calf thymus DNA/ml in the hybridization solution described above. Hybridization and autoradiography were carried out in the same procedure for DNA blots.

Nuclease S1 analysis

Polyadenylic acid-containing RNA was hybridized with the *Acc*I-H fragment of Ad12 DNA in a solution containing 80% formamide, 0.4 M NaCl, 0.04 M PIPES (pH 6.4), and 0.001 M EDTA (3) at 53 ℃ (15) essentially by the method of Berk and Sharp (1). RNA-DNA hybrids were digested with nuclease S1 as described previously (1, 15). For two-dimensional S1 analysis, the nuclease S1-resistant RNA-DNA hybrids were first electrophoresed in a neutral agarose disc gel (2 mm in diameter). The gel cylinder was equilibrated for alkaline electrophoresis buffer, and set on top of a second alkaline agarose slab gel.

After denaturation and neutralization of a neutral gel and neutralization of one- and two-dimensional alkaline gels, DNA fragments were transferred to nitrocellulose membrane filters, hybridized labeled probes, and autoradiographed as described above for DNA blot analysis.

RESULTS

The persistence of viral sequence in HY cells has been shown by hybridization and reassociation experiments (20). Southern blot analysis shown in Fig. 2-A demonstrated the integration of the viral sequence in a large fragment produced by *Hind*III digestion of high-molecular-weight cellular DNA. The same results were obtained by digestion with various enzymes including *Bam*HI, *Eco*RI, *Sma*I, *Bcl*I,

*Cla*I, *Hpa*I, *Sst*I, *Sst*II, and *Xba*I (figure not shown). When cellular DNAs were cleaved with *Acc*I, the viral sequence was found in two DNA fragments of 2.4 kb and approximately 19 kb (Fig. 2-A and Fig. 3, lane 3). The integration pattern of viral sequence was indistinguishable with different cell lines. Even after digestion with *Hae*III which often cleaves DNA in general, and has one cleavage site in the *Acc*I-H fragment, the pattern was identical, except for the presence of an extra minor band in HY2-1 and HY7-1.

Several enzymes cleaved the integration site into two to three portions as well as *Acc*I. Two fragments of 5.1 kb and approximately 19 kb were produced by *Pst*I

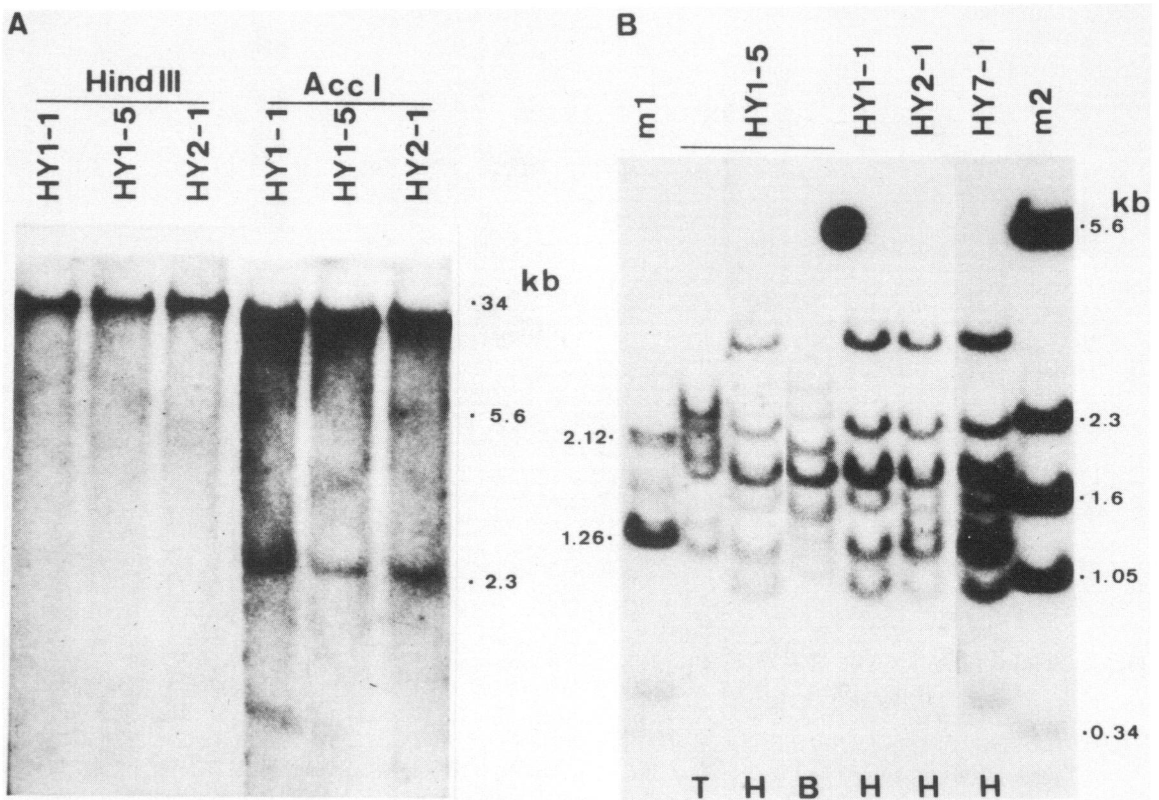

Fig. 2 Southern blot analysis of integrated viral sequence in clonal lines of transformed cells.

DNA blots were prepared, hybridized labeled *Acc*I-H fragment and autoradiographed as described in Materials and Methods. (A) 10 μg of high-molecular-weight DNAs from indicated cell lines were cleaved with *Hin*dIII or *Acc*I. Size markers used were Ad12 DNA (34 kb), Ad12 *Eco*RI-C fragment (5.6 kb) and Ad12 *Hin*dIII-G fragment (2.3 kb). (B) 15 μg of high-molecular-weight DNAs from indicated cell lines were cleaved with *Taq*I(T), *Hae*III(H), or *Bst*NI(B). Size markers used were (m1) *Taq*I digest (2.12 kb) and *Hae*III digest (1.26 kb) of pASC-1 DNA, and (m2) Ad12 *Eco*RI-C, *Hin*dIII-G, *Acc*I-H fragments and subfragments (1.05 kb, 0.34 kb) of *Acc*I-H cleaved with *Taq*I and *Hae*III.

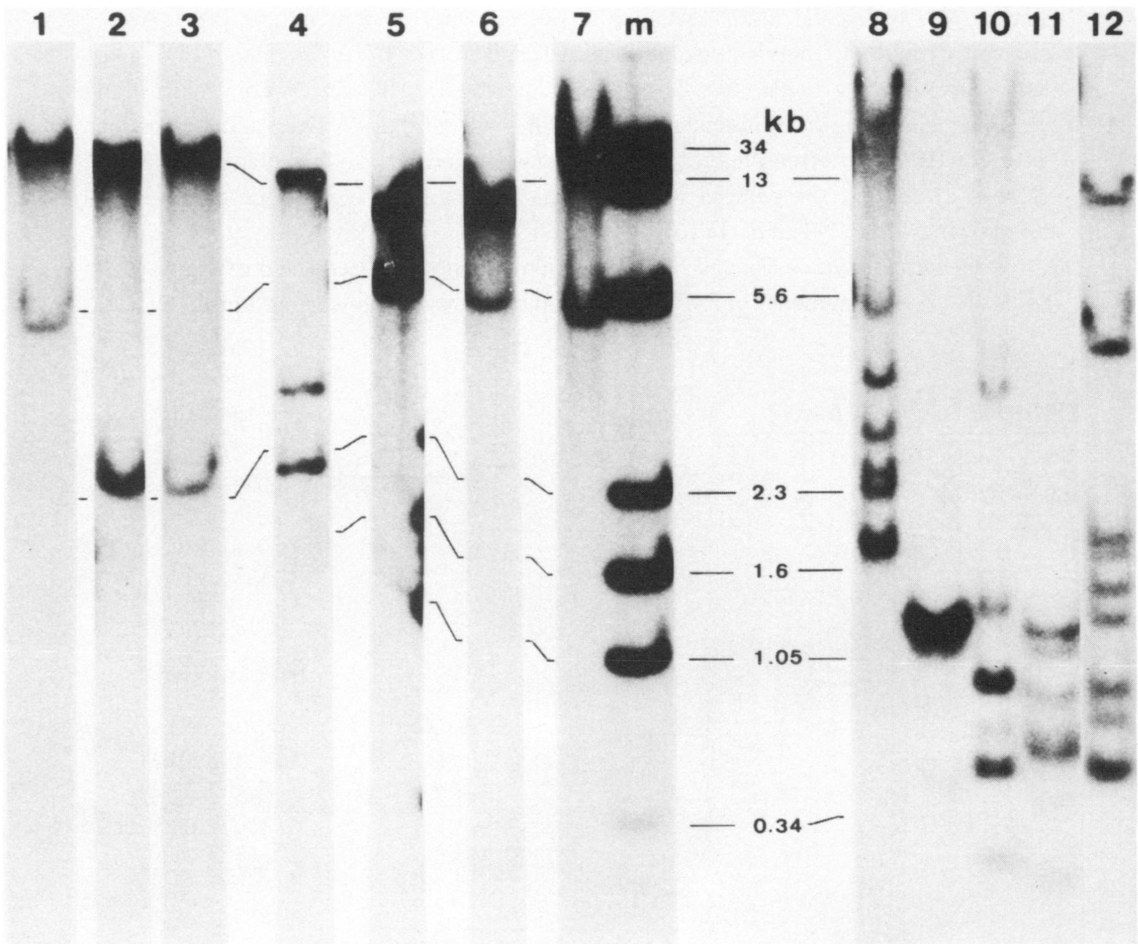

Fig. 3 Southern blot analysis of HY1-5 cell DNA.

High-molecular-weight DNA (12.5 μg) from HY1-5 cells was cleaved with restriction endonucleases (1) *Pst*I, (2) *Pst*I+*Acc*I, (3) *Acc*I, (4) *Acc*I+*Hinc*II, (5) *Ava*I+*Hinc*II, (6) *Ava*I, (7) *Hinc*II, (8) *Hga*I, (9) *Hga*I+*Bst*NI, (10) *Hap*II, (11) *Alu*I, and (12) *Pvu*II. Size markers (m) included the same DNAs as shown in Fig. 2, except for pAdC22 DNA (13 kb) linearlized with *Bam*HI. DNA blots were prepared, hybridized labeled *Acc*I-H fragment, and autoradiographed as described in Materials and Methods.

(Fig. 3 lane 11), three of 5.2 kb, 9.0 kb and 10.0 kb by *Ava*I (Fig. 3, lane 5), two of 5.1 kb and approximately 19 kb by *Hinc*II (Fig. 3, lane 7), and three of 6.8 kb, 12.2 kb and approximately 17 kb by *Bal*I (figure not shown). Since these enzymes have no restriction sites within the *Acc*I-H fragment, they should cleave spacer DNA segments between integrated viral DNA copies.

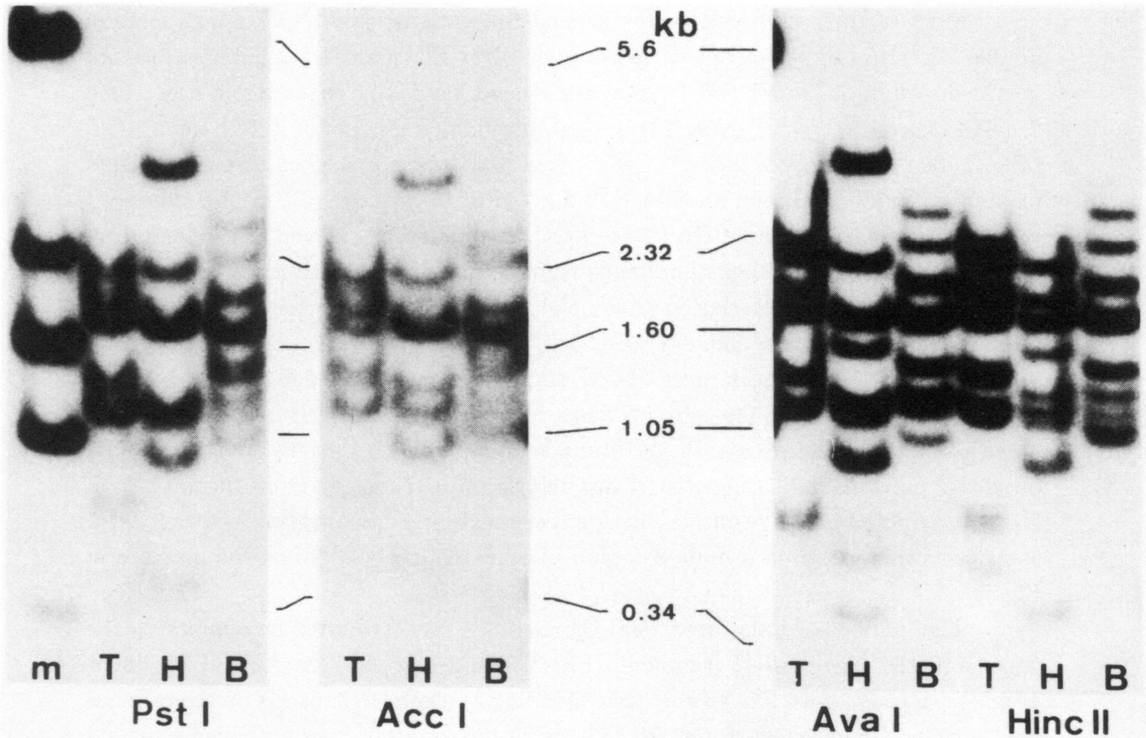

Fig. 4 Double-digestion analysis of HY1-5 cell DNA.

High-molecular-weight DNA (12.5 μg) from HY1-5 cells was cleaved with a pair of restriction endonucleases as shown in the figure. *Taq*I (T), *Hae*III (H) and *Bst*NI (B) cleave once within the *Acc*I-H fragment as shown in Fig. 1, while *Pst*I, *Acc*I, *Ava*I and *Hinc*II have no restriction site within the viral DNA fragment. Size markers used were the same as in Fig. 2. DNA blots were prepared, hybridized labeled *Acc*I-H fragment, and autoradiographed as described in Materials and Methods.

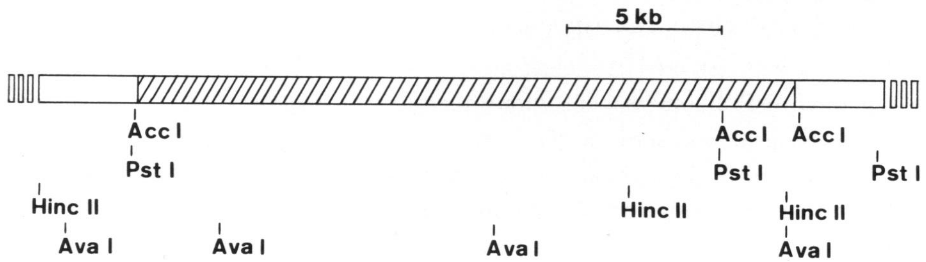

Fig. 5 Partial restriction map around integrated viral sequences in HY1-5 cell DNA.

All of the integrated viral sequences, including at least 8 copies of *Acc*I-H sequence of Ad12 DNA, were located as a cluster within the hatched region of 22 kb between the *Acc*I sites indicated in the figure. Cleavage sites of restriction endonucleases which have no sites within the viral sequence are shown based on the data represented in Figs. 2, 3, and 4. Most viral DNA copies are dispersed by spacer segments in this region.

Most of their restriction sites around the integration site were mappped by double digestion experiments. The smaller *Acc*I fragment from integration site was included in the small *Pst*I fragment as shown by double digestion in Fig. 3 lane 2. *Acc*I cleaved the small *Hinc*II fragment into two fragments of 2.1 kb and 3.0 kb (Fig. 3, lane 4). Double digestion with *Ava*I and *Hinc*II produced three fragments of approximately 5 kb and another of 9.2 kb (Fig. 3, lane 5). By double digestion with *Acc*I or *Pst*I, the same *Hae*III or *Bst*NI fragment was cleaved as shown in Fig. 4. The resulting double digestion bands were similar in size with a small difference of 0.1 kb. Thus, the restriction sites of *Acc*I and *Pst*I within the integration site were located in the close vicinity (Fig. 5).

*Hinc*II cleaved the largest *Hae*III fragment which was not cleaved by *Pst*I, *Acc*I or *Ava*I (Fig. 4). Thus *Hinc*II has a restriction site apart from *Pst*I, *Acc*I, or *Ava*I inside the integration site. *Ava*I made only minor alterations on *Taq*I, *Hae*III or *Bst*NI pattern, indicating that it has restriction sites apart from those of *Pst*I, *Acc*I, or *Hinc*II. As a result of these experiments, the integration site of viral sequence was located in a limited region of approximately 22 kb as summarized in Fig. 5.

The number of integrated viral DNA copies was estimated by comparing the density of the *Hga*I–*Bst*NI fragment (Fig. 3, lane 9) or the *Taq*I–*Bst*NI fragment (Fig. 6, panel B) with that of the corresponding marker DNA bands on the original photographs. The *Hga*I–*Bst*NI band was approximately twice as dense as its marker band of similar size at 6 copies equivalent/diploid DNA quantity. The *Taq*I–*Bst*NI band density was approximately a half compared to marker DNA bands of the same size at 20 copies equivalent. Thus approximately 10 copies of viral DNA fragment were integrated in a limited region of high-molecular-weight DNA in HY1-5 cells.

When HY1-5 cell DNA was digested with *Hga*I and *Bst*NI, most of viral sequence was detected in a band at 1.2 kb (Fig. 3, lane 9). Since it is identical to the *Hga*I–*Bst*NI subfragment within the *Acc*I–H fragment, most viral DNA copies carry the sequence from the recognition site of *Hga*I at 169 at the left end to that of *Bst*NI at 1390 at the right end in a colinear fashion. As *Hap*II produced a relatively dense band of 0.5 kb (Fig. 3, lane 10), and *Alu*I of 0.6 kb (Fig. 3, lane 11), at least some copies brought up to position 152 at the left end and up to position 1506 at the right end. These results indicated the presence of all the transcription unit of ElA gene in these cells.

Up to ten bands were detected by cleavage with *Taq*I, *Hae*III, *Bst*NI (Fig. 2–B), *Hga*I (Fig. 3, lane 8) or *Pvu*II (Fig. 3, lane 12). Since these enzymes have one or two cleavage sites in the *Acc*I–H fragment, these results indicate the diversity of spacer sequences between most of the viral DNA copies. This point

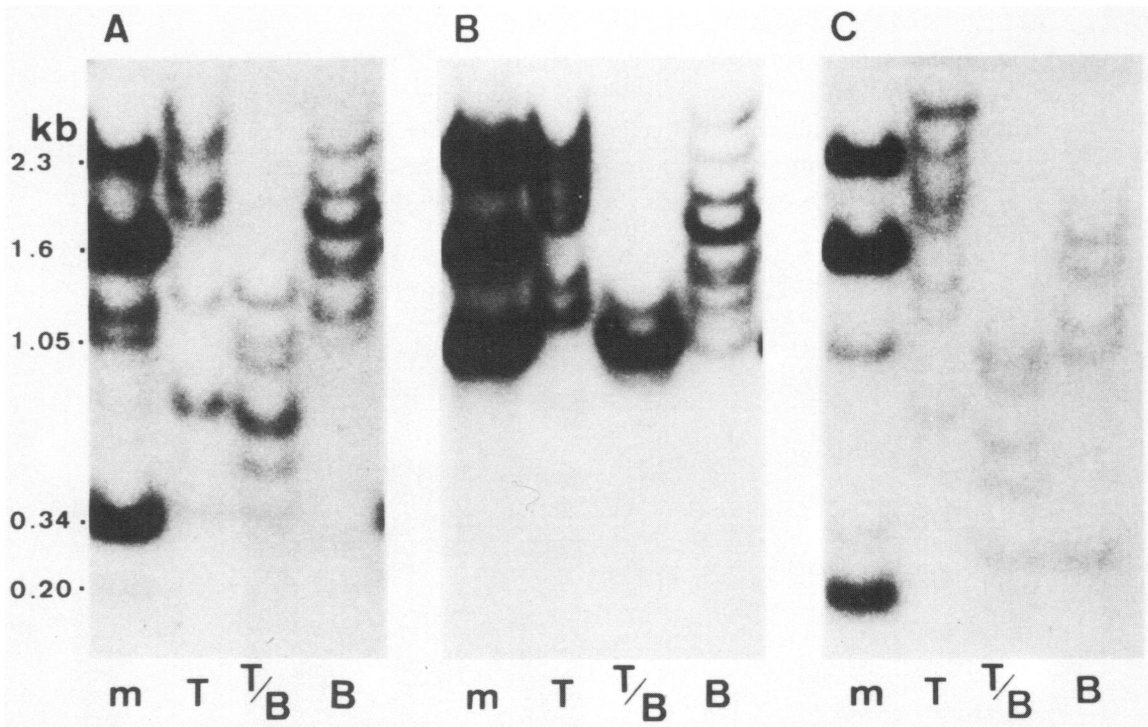

Fig. 6 Analysis of flanking structure of integrated viral DNA copies in HY1-5 cell DNA.
High-molecular-weight DNA (15 μg) from HY1-5 cells was cleaved singly with *Taq*I(T) and *Bst*NI(B) or in combination with these enzymes (T/B). Size markers used were *Hin*dIII-G (2.3 kb), *Acc*I-H (1.6 kb) and subfragments (1.05 kb, 0.34 kb, 0.20 kb) of *Acc*I-H cleaved with *Taq*I and *Bst*NI. Labeled probes used in hybridizing DNA blots were (A) leftmost 0.34 kb subfragment of *Acc*I-H produced by *Taq*I cleavage, (B) internal 1.05 kb subfragment of *Acc*I-H produced by *Taq*I and *Bst*NI cleavage, and (C) rightmost 0.20 kb subfragment of *Acc*I-H produced by *Bst*NI. Restriction sites of these enzymes are shown in Fig. 1. Panels A and C were autoradiographed for 2 weeks while panel B was for one day.

was further analyzed by hybridization of the DNA blots of *Taq*I and *Bst*NI double digestion products of HY1-5 cell DNA to three subfragments of *Acc*I-H prepared by *Taq*I and *Bst*NI cleavage. The internal probe detected a few minor bands besides the major band of intact size as shown in Fig. 6 panel B. When identical DNA blots were hybridized to external probes of the leftmost *Taq*I fragment (0.34 kb) or the right most *Bst*NI fragment (0.20 kb), six or five discrete bands were observed, respectively (Fig. 6, panel A and C lanes T/B). These results indicated the variety of the flanking structure at both left and right ends of integrated viral sequence. Some of *Taq*I or *Bst*NI bands were not detected with the leftmost or rightmost probe as shown in Fig. 6 panels A and C. It suggested the presence of minor variety in the

end points of the integrated viral DNA copies.

Sl mapping experiments revealed the presence of normal ElA mRNA species in all HY cells analyzed (Fig. 7, panel B). Out of four typical ElA mRNA species synthesized in lytically infected cells as shown in Fig. 1, two species transcribed from the downstream start site were detected. These are composed of 0.62 kb, 0.53 kb and 0.31 kb exons as shown in a two-dimensional gel (Fig. 7, panel A).

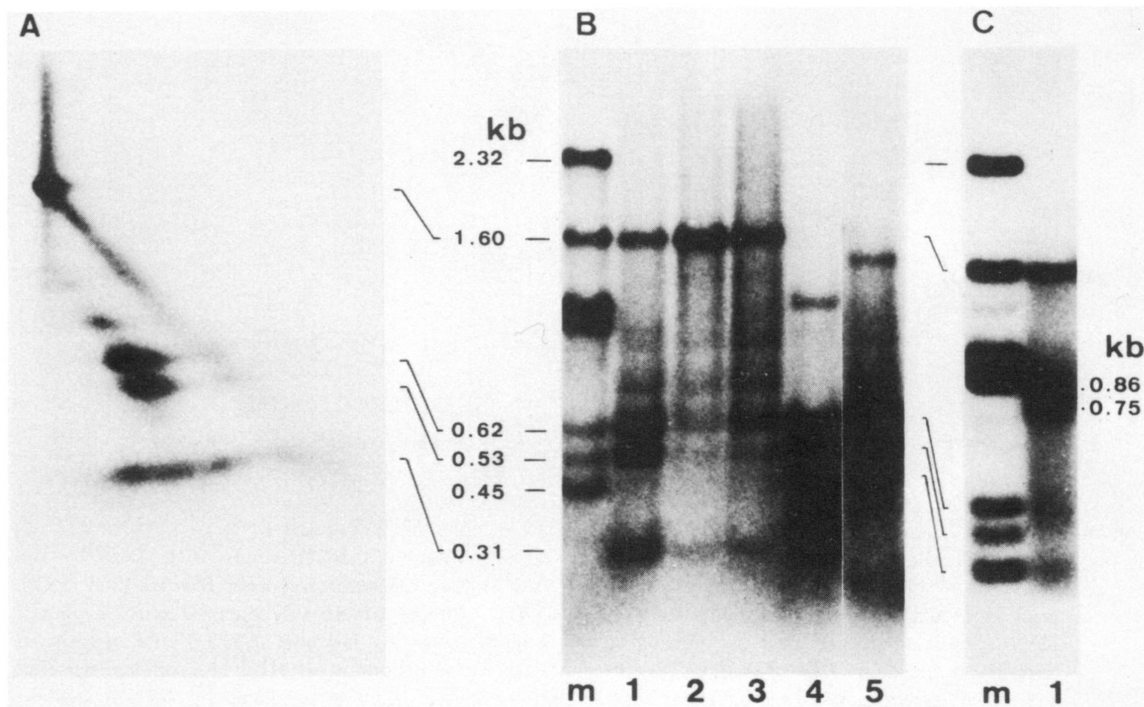

Fig. 7 Nuclease Sl analysis of mRNAs prepared from HY1-5, HY2-1 and HY7-1 cells.
A. Polyadenylic acid-containing RNA (10 μg) from HY1-5 cells were hybridized *Acc*I-H fragment, treated with nuclease Sl and subjected to neutral (left to right) and alkaline electrophoresis (top to bottom) to make two-dimensional DNA blot as described in Materials and Methods. Labeled *Acc*I-H fragment was used as the hybridization probe. B. Polyadenylic acid-containing RNAs from (1, 4, 5) HY1-5 (5, 10, 10 μg, respectively), (2) HY2-1 (5 μg) and (3) HY7-1 (5 μg) were hybridized (1, 3, 5) *Acc*I-H fragment, subfragments of *Acc*I-H cleaved with (4) *Hae*III and (5) *Bst*NI. After nuclease Sl digestion and alkaline electrophoresis, DNA blot was prepared and hybridized to labeled *Acc*I-H fragment. Size markers were *Hind*III-G (2.32 kb), *Acc*I-H (1.6 kb) and subfragments of *Acc*I-H cleaved with *Ava*II (0.62 kb, 0.53 kb, 0.45 kb) and *Taq*I+*Bst*NI (1.05 kb, 0.34 kb; not specified in the figure). C. Polyadenylic acid-containing RNA (5 μg) from HY1-5 cells was hybridized (1) *Acc*I-H fragment, and digested with nuclease Sl. Markers were the same as described in the legend to panel B. After electrophoresis on a neutral gel, DNA blot was prepared, hybridized to labeled *Acc*I-H fragment as shown in Materials and Methods.

Joint molecules of 0.62kb+0.31kb and 0.53kb+0.31kb were detected as 0.86kb and 0.75 kb bands in a neutral gel (Fig. 7, panel C). The two-dimensional gel also detected a minor mRNA species transcribed from upstream start site as a 0.76 kb exon. These mRNA species were detected by RNA blot analysis of polyadenylic acid-containing RNA prepared from HY1-5, HY2-1 and HY7-1 cells (data not shown).

DISCUSSION

The structure and state of integrated viral genome have been analyzed in various cell lines established by adenovirus transformation (5, 7, 14, 17, 24, 25). These studies have revealed that viral DNAs were integrated, with deletions of terminal regions to various extents, in various sites of cellular DNA. Thus, no specific integration site has been revealed either in cellular or adenoviral sequence. These features are also found in the case of transformed cells induced by transfection of *Hin*dIII-G fragment of Ad 12 or *Hin*dIII-IJ fragment of Ad 7 (16, Sugisaki *et al.*; Kudo *et al.*; Yoshida *et al.*; manuscripts in preparation). These cells contain all the ElA gene and a portion of ElB gene which includes the entire coding region for the 19-20 kb protein, one of the ElB gene products.

In the present paper, we analyzed the structure of viral DNA and the expression of viral messages in HY cell lines which were previously established by transfection of the *Acc*I-H fragment. Southern blot analysis showed that approximately 10 copies of the viral DNA fragments were integrated in HY1-5 cells. This agrees with the previous results of reassociation kinetic analysis of HY1 cells (20). It is also shown by the above results that all the viral sequences are localized within a limited region of approximately 22 kb which is represented by two adjacent *Acc*I fragments (Fig. 5). Most of the viral DNA copies span from the *Hga*I site at position 169 to the *Bst*NI site at position 1390. Detection of the *Hap*II fragment from the positions 154-657 and the *Alu*I fragment from the positions 908-1504 further expands the junction points of viral DNA to the flanking sequences. Therefore at least some copies of integrated viral sequence cover the entire genetic information to express the ElA gene functions.

Synthesis of ElA mRNA species of normal structure was demonstrated in HY 1-5, HY 2-1 and HY 7-1 cells by Sl mapping and RNA blot analysis. It excludes the possibility that the incompleteness of transformed phenotype of these cells is due to the lack or alteration of a portion of ElA gene. Two species transcribed from the downstream start site were dominant. One of two possible species from the upstream start site was detected in these cells, as was the case with other transformed cells GYl and CYl (15). It seems an intrinsic feature of derivatives of rat 3Yl cells, since four ElA mRNA species are synthesized in human KB cells

infected with Ad12 (15).

Most of the integrated viral DNA were flanked by spacer sequences. The spacer segments have various sizes as indicated by the discrete multiple bands produced by digestion with *Taq*I, *Hae*III, *Bst*NI, *Hga*I and *Pvu*II. It is further elucidated by blot analysis of *Taq*I/*Bst*NI double digestion products with subfragment probes. Distribution of cleavage sites of *Acc*I, *Pst*I, *Hinc*II and *Ava*I suggests their sequence variety, likewise. These complicated features are similar to the results of analyses of adenoviral DNA integration in many transformed cells (5, 7, 14, 17, 24, 25, Sugisaki *et al.*; Kudo *et al.*; Yoshida *et al.*; manuscripts in preparation), and render it difficult to understand the mechanism of integration. It is, however, noteworthy that the state of integrated viral sequence was nearly identical in three cell lines established from independent transformed foci. It suggests that the integration and possible rearrangement took place in a similar manner through the transformation process of independent cells.

ACKNOWLEDGEMENTS

We wish to thank Dr. H. Shimojo, Institute of Medical Science, University of Tokyo, for the donation of pASC-1. We are also grateful to Miss N. Ono-oka and Mr. M. Ishino for their help in preparing this manuscript. This work was supported in part by Grants-in-Aid for Cancer Research and for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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