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Structure and Expression of the Integrated Viral DNA in a Rat Cell Line Transformed by the Left-terminal 7.8% Fragment of Adenovirus Type 7 DNA

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SUMMARY

Integrated viral DNA sequences and cDNA copies of viral mRNAs were cloned from 7IJY1-1, a rat 3Y1 cell line transformed by the adenovirus 7 DNA fragment (*Hin*dIII-I•J, 0 to 7.8% map position). The 7IJY1-1 cell line contained most of the sequences of the *Hin*dIII-I•J, and its molecular arrangement in the rat cell DNA was complicated, exhibiting linkages among different pieces of the *Hin*dIII-I•J in a head to tail or a head to head orientation. Analysis of the cloned cDNA indicated that some of the fused viral DNAs serve as transcription templates for E1B-E1A fusion mRNAs which were found especially in 7IJY1-1 cells. *In vitro* translation of hybridization-selected viral mRNAs showed that E1B-E1A fusion mRNAs direct the synthesis of the E1B gene-encoded 21,000-dalton protein. These results show that in the 7IJY1-1 cell line, linkage structure of the integrated *Hin*dIII-I•J permit transcription of E1B-E1A fusion mRNAs and expression of the E1B gene-encoded 21,000-dalton protein.

Key words: Ad7 transforming DNA, Integration, Viral RNA, Viral protein

INTRODUCTION

The transforming gene of human adenoviruses 7 (Ad7) has been localized within the left-terminal 11.2% of the viral genome, in a segment corresponding to the early gene region 1 (E1) (1, 2, 3) . The E1 region can be divided into two transcriptional units: the E1A from map position 1.3 to 4.4% and the E1B from map position 4.5 to 11.2% (4, 5). The E1A region encodes three coterminal, but differently spliced mRNAs of 1.1 kilobase (1.1 kb), 0.9 kb and 0.6 kb which have a coding potential for proteins with theoretical molecular weights of 28,000 (E1A-28

K), 24,000 (E1A-24 K), and 6,300 (E1A-6.3 K), respectively (4, 5, 6). The E1B region encodes two major mRNAs of 1.1 kb and 2.5 kb with a common terminal but different splices (8, 45). The 1.1 kb mRNA directs the synthesis of a protein of 21,000 (E1B-21 K), whereas the 2.5 kb mRNA directs both proteins of 21,000 and 55,000 (E1B-55 K)(4). *In vitro* transformation studies (1) showed that the left-terminal 4.5% fragment of the viral genome (Ad7 *Bgl*II-H) comprising the E1A region induces partially or incompletely transformed cells. On the other hand, the left-terminal 7.8% fragment of the viral genome (Ad7 *Hin*dIII-I•J)induces fully or completely transformed cells, exhibiting colony formation in soft agar culture and oncogenicity in transplanted rats (2). These results suggest that gene product(s) specified in the 5'-terminal half (4.5-7.8%) of the E1B region are required for induction of transformed cells with characteristic transforming phenotypes. For the expression of the truncated E1B gene, the polyadenylation site for the E1B gene should be complemented by others elsewhere at the integrated viral DNAs or their flanking cellular DNAs. Our previous RNA mapping results (5) showed that rat 3Y1 cell lines transformed by the Ad7 *Hin*dIII-I•J synthesized the 1.1 and 0.9 kb E1A mRNAs and two or three unusual viral mRNAs, and that the unusual species appeared to be E1B-E1A fusion mRNAs transcribed from both regions of the E1B and the E1A.

In this paper, we have cloned integrated viral DNA sequences and cDNA copies to viral mRNAs from a 7IJY1-1 cell line, a rat 3Y1 cell line transformed by the Ad7 *Hin*dIII-I•J. The relationship between molecular arrangement and transcription of the integrated *Hin*dIII-I•J was analyzed. We have also analyzed virus-specific proteins synthesized in 7IJY1-1 cells using *in vitro* translation of mRNAs selected by hybridization with viral DNA. The data showed that the *Hin*dIII-I•J was located at the two fragments of the *Eco*RI-cleaved cell DNA in a tandemly joined manner and that some of the joined viral DNAs were transcribed into the E1B-E1A fusion mRNAs which predominantly directed the synthesis of the E1B gene-encoded protein with an apparent molecular weight of 16,000 (16 K) and the E1A gene-encoded 45 K protein.

MATERIALS AND METHODS

Cells and virus

Ad7, Grider strain, was propagated in human KB cells and viral DNA was isolated from purified virions as described by Green and Pina (7, 8). KB cells were cultured as a monolayer in Eagle minimal essential medium (MEM) supplemented with 10% calf serum. The 7IJY1-1 cell line is a subclone of the 7IJY1 cells (2) which was established from a single cell colony grown in 0.33% agar medium after transfection of a rat 3Y1 cell line with the Ad7 *Hin*dIII-I•J. The 7IJY1-1 cells were

cultured as a monolayer in MEM containing 0.1 mM $CaCl_2$ supplmented with 10% fetal calf serum.

Southern blot hybridization

Total DNA was prepared from the 7IJY1-1 and 3Y1 cell lines, as described previously (9). The DNA was digested with excess amounts of restriction enzymes, and digestion products were electrophoresed with 0.9% agarose gels. The DNA was denatured *in situ*, transferred onto nitrocellulose filters (Schleicher and Schuell, BA85), and immobilized by baking at 80℃ for 3 h (10). Hybridizations were carried out at 45℃ for 24 h in 50% formamide-0.4 M NaCl-0.1 M Tris (pH 8.0)−5 mM EDTA and [^{32}P]−DNA nick-translated to a specific activity of 1-2× 10^8 cpm/μg (11). The filters were washed four times in 50% formamide-2×SSC (1×SSC is 0.15 M NaCl plus 0.015M sodium citrate) -0.5% sodium dodecyl sulfate (SDS) and twice in 0.2×SSC−0.1% SDS at 50℃. Dried filters were exposed for autoraidography on Kodak XRP-1 Xray films with Dupont intensifying screen at −70℃.

Molecular cloning of integrated viral DNAs into a plasmid pBR322

The cell DNA (300-500 μg) was digested with *Hin*dIII, and the digestion products were electrophoresed with a size marker (*Bam*HI fragments of Ad7 DNA) in a preparative 1.0% agarose gels. After standing with 0.5 μg of ethidium bromide per m*l*, agarose gel fractions containing viral DNA sequences, previously assayed for the presence of viral sequences by Southern blot hybridization analysis, were cut out. DNAs were eluted electrophoretically from gel blocks using ISCO Model 1,750 and purified by phenol extractions and two cycle applications on small columns of DEAE cellulose. The *Hin*dIII fragments of the cell DNA were ligated with the *Hin*dIII-linearlized and dephosphorylated pBR322 DNA as described by Maniatis *et al.* (12). The ligated DNAs were introduced to *Escherichia coli* strain HB101 pretreated with 0.1 M $CaCl_2$ as described by Curtis *et al.* (13). Ampicillin-resistant colonies were screened for the presence of viral DNA sequences by the high density colony hybridization technique (14), using nick-translated Ad7 [^{32}P] *Sal*I-C (0−17.5%) as a probe.

Cloning of DNAs complementary to virus-specific mRNAs

Cytoplasmic RNA was isolated from 7IJY1-1 cells as described by Zieve and Penman (15). Polyadenylic acid-containing RNA (poly A RNA) was selected by passage through a oligo (dT)-cellulose column and fractionated by a centrifugation through 35 m*l* of 5 to 20% (W/V) sucrose density gradient in 50% formamide-0.1 M LiCl-1mM EDTA-0.1% (W/V) SDS-10 mM Tris (pH 8.0) at 25,000 rpm for 40 h at 20℃, using a Beckman SW 27 rotor. The RNA of 1.5 to 2.2 kb in sizes were

precipitated with ethanol and subjected to cDNA synthesis. The first strand of cDNA was synthesized by the method described by Perricaudet *et al.* (16). The second strand of cDNA was synthesized in the reaction mixture containing 40 mM Tris (pH 7.5), 7 mM $MgCl_2$, 50 mM KCl, 1 mM each of dATP, dCTP, dGTP and TTP, 4 mM dithiothreitol, and 75 units of the Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer Mannheim Corp.) The DNA was recovered in the same manner as the synthesis of the first strand (16) and digested with 5 units of S1 nuclease (Bethesda Research Laboratories, Inc.). DNA was fractionated by centrifugation through 5–20% sucrose density gradient as described by Maniatis *et al.* (12). DNAs with the length of 0.5 kb or over were pooled and tailed with approximately 20 dC nucleotides by calf thymus terminal transferase as described (17). The dC-tailed DNA was annealed with the *Pst*I-linearized and dG-tailed pBR322 (Bethesda Research Laboratory Corp.) at a concentration of 1 μg DNA per m*l* with a molar ratio of 1 to 1 and introduced into $CaCl_2$-treated *Escherichia coli* strain HB101. Tetracycline-resistant colonies were screened for the presence of viral DNA sequences by high density colony hybridization (13) using nick-translated Ad7 [^{32}P] *Sal*I-C (0–17.5%).

Hybridization selection and in vitro translation of viral RNAs

Using the method described by Halbert *et al* (18), virus specific mRNAs were selected by hybridization with restriction fragments of Ad7 DNA immobilized on nitrocellulose filters. Filters bearing 1 μg of restriction fragments were incubated at 45℃ for 5 h with 100 μg of cytoplasmic poly A RNA in a buffer containing 20 mM Pipes (pH 6.4), 0.4 M NaCl, 1 mM EDTA and 50% formamide. The filters were washed 5 times with 2×SSC−0.5% SDS, twice with 0.2×SSC−0.1% SDS and twice with 1 mM EDTA (pH 7.5) at 50℃. Then, the filters were soaked in distilled water, heated in boiling water bath for 2 min and cooled quickly. The filters were removed, and the eluted RNA was precipitated by an addition of ethanol with 10 μg of calf liver tRNA (Boehringer Mannheim Corp.). Selected RNA was translated in a rabbit reticulocyte lysate pretreated with micrococcal nuclease (a gift from Yutaka Kogo, Sapporo Medical College, Sapporo, Japan). Proteins synthesized in the presence of [^{3}H]-leucin were separated in SDS-containing polyacrylamide gels by the method of Laemmli (19), and the gels were processed for fluorography (20).

DNA sequencing

DNA sequence determination was performed as described by Maxam and Gilbert (21).

RESULTS

Southern blot hybridization analysis of the integrated HindIII-I•J

Integration pattern of the *Hin*dIII-I•J in the cell DNA was analyzed by Southern blot hybridization (Fig. 1). Total DNAs from 7IJY1-1 cells and untransformed parental rat 3Y1 cells were digested with restriction endonucleases, electrophoresed in agarose gels, and transferred onto nitrocellulose filters. DNA fragments containing viral sequences were detected by hybridization with two probes of [^{32}P]-labeled viral DNA ; E1A region-specific DNA (*Hin*dIII-I : 0-4.0%, panel A) and E1B region-specific DNA (*Hin*dIII-J/*Bgl*II : 4.5-7.8%, panel B). The *Eco*RI, an enzyme with no cleavage site in the *Hin*dIII-I•J, generated two DNA fragments (13 kb and 15 kb) which hybridized with both probes of E1A and E1B. Next, the cell DNA was digested with *Hin*dIII and *Bgl*II, enzymes with a single

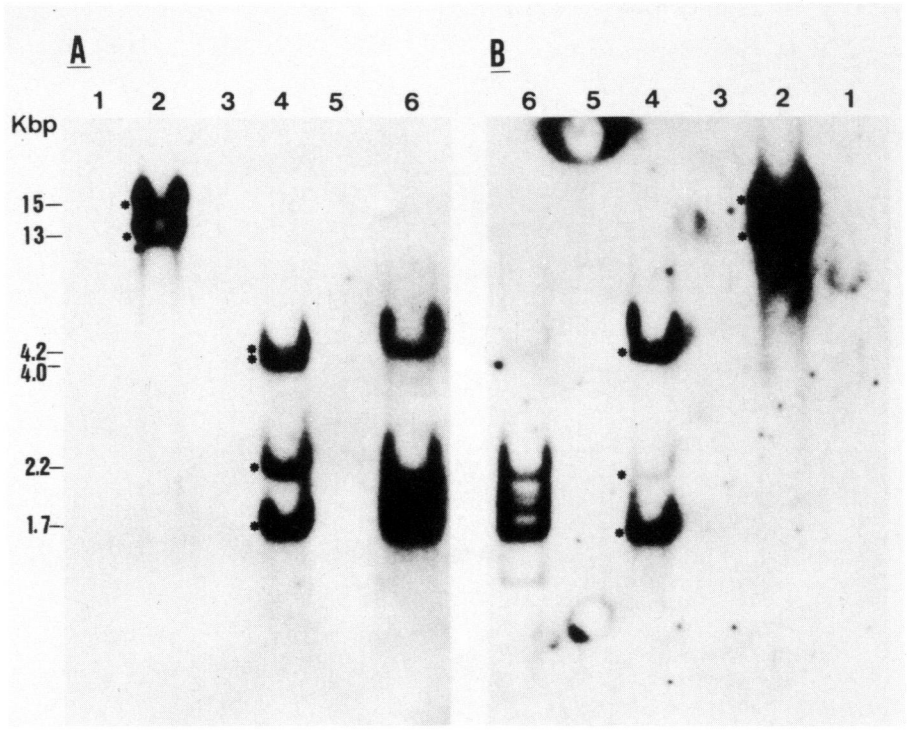

Fig. 1 Viral DNA sequences in 7IJY1-1 cells. Fifteen micrograms of high molecular weight DNA from 7IJY1-1 cells (lanes 2, 4, and 6) and untransformed 3Y1 cells (lanes 1, 3, and 5) were cleaved with *Eco*RI (lanes 1 and 2), *Hin*dIII (lanes 3 and 4) and *Bgl*II (lanes 5 and 6), electrophoresed in a 1.0% agrose gel, transferred to a nitrocellulose filter, and hybridized with Ad7 [^{32}P] *Hin*dIII-I (0-4.0%, panel A) or [^{32}P] *Hin*dIII-J/*Bgl*II (4.5-7.8%, panel B). The size determination was carried out using Ad12 *Hin*dIII fragments as a marker.

cleavage site in the *Hin*dIII-I•J. The *Hin*dIII cleavage yielded four DNA fragments (4.2, 4.0, 2.2, 1.7 kb), three of which hybridized with both probes of E1A and E1B. The *Bgl*II produced six fragments, five of which also hybridized with both probes (Fig. 1). When the *Hin*dIII-I•J is present in a tandemly joined manner, the *Hin*dIII or *Bgl*II cleavage should yield DNA fragments hybridized with both probes. These results lead to the simple understanding that most of the integrated *Hin*dIII -I•J are joined together and located at the two fragments of the *Eco*RI-cleaved cell DNA. Shiroki *et al* (22) indicated by cotransfection experiments that the expression of the E4 gene is required for the establishment of the Ad12 El-transformed rat 3Y1 cell lines which are capable of growing in soft agar. To confirm the presence or the absence of the E4 region sequences, the 7IJY1-1 cell DNA was analyzed by Southern blot hybridization using [^{32}P]-labeled DNA fragment (92.4-97%)

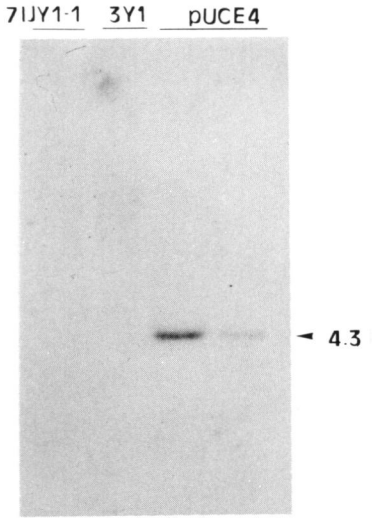

Fig. 2 Southern blot hybridization analysis of the E4 region sequences. High molecular weight DNA from the 7IJY1-1 and 3Y1 cells (15μg each) and pUCE4 DNA (10pg, 2.5pg) were cleaved with *Hin*dIII, electrophoresed in a 1.0% agarose gel, transferred to a nitrocellulose filter, and hybridized with [^{32}P] DNA (92.4 -97%) spanning the Ad7 E4 region. The pUCE4 is a plasmid pUC19 carrying the *Sma*I and *Bam*HI digested fragment (92.4-97%) of Ad7 DNA.

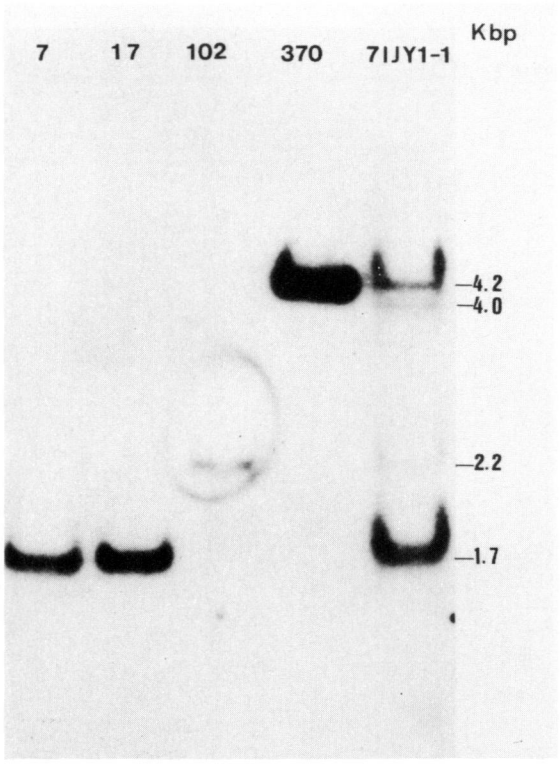

Fig. 3 *Hin*dIII cleavage patterns of the viral DNA cloned from the 7IJY1-1 cell DNA. Plasmid DNAs (clones 17, 7, 102, and 370) and the 7IJY1-1 cell DNA were cleaved with *Hin*dIII, electrophoresed in a 1.0% agarose gel, transferred to a nitrocellulose filter, and hybridized with Ad7 [^{32}P] *Sal*I-C (0-17.5%).

spanning the Ad7 E4 region as a probe (Fig. 2). A positive signal of hybridization could not be observed within a limit of sensitivity at 0.1 copy of the E4 region fragment per haploid genome, indicating no sequences of the E4 region in 7IJY1-1 cells.

Molecular cloning analysis of the integrated HindIII-I•J

The *Hin*dIII-cleaved cell DNA fragments with lengths of 1.7, 2.2, 4.0 and 4.2 kb, previously assayed for the presence of viral sequences by Southern blot hybridization analysis (Fig. 1), were prepared from 7IJY1-1 cells. These fragments were inserted into the *Hin*dIII cleavage site of pBR322 DNA. Approximately 50,000 ampicillin-resistant colonies per each fragment were screened for the presence of viral DNA sequences. Fragments with sizes of 1.7 kb (two different clones 7 and 17), 2.2 kb (clone 102), and 4.2 kb (clone 370) were cloned. These cloned DNAs were cleaved out from the vector sequences with *Hin*dIII and were indistinguishable in electrophoretic mobilities from the viral sequence-containing *Hin*dIII fragments generated from the cell DNA (Fig. 3). Cloned fragments were also digested with other restriction enzymes, and cleavage sites were mapped (data not shown). A comparison of cleavage sites between the cloned DNAs and their parental *Hin*dIII -I•J revealed partial structure of the integrated viral DNA (Fig. 4). All the cloned DNAs were of viral origin and consist of linkages among two or three pieces of the *Hin*dIII-I•J. In clone 17, the 1.7 kb insert contains sequences derived from the right - and left-hand pieces (4.0-7.0%, 2.2-4.0%) of the *Hin*dIII-I•J. These DNA sequences were joined in a head to tail manner between the same DNA strands. In clone 7, two right hand pieces (4.0-5.2%, 4.0-7.3%) of a *Hin*dIII-I•J are joined in a head to head manner between the different strands. In clone 102, three pieces (4.0 -6.7%, 3.1-3.8%, 4.0-7.1%) of the *Hin*dIII-I•J are joined in a head to tail and a head to head manner. In clone 370, four pieces (4.0-7.1%, 0.2-3.8%, 4.1-6.4%, 0.88 -4.0%) of the *Hin*dIII-I•J are joined in a head to head and a head to tail manner. Thus, structural analysis of the cloned viral DNAs indicated that molecular arrangement of the integrated *Hin*dIII-I•J are complicated, exhibiting linkages among different pieces of the *Hin*dIII-I•J in a head to tail or a head to head orientation.

Nucleotide sequences at the junction sites of viral DNAs

The nucleotides of either *l*- or r-strand around the junctions of viral DNAs, together with two joining parental DNA sequences adjacent to each junction were shown in figure 5. All the seven junctions contain no sequences derived from rat cell DNA or salmon sperm DNA (a carrier DNA when the *Hin*dIII-I•J was transfected). At each of the seven junctions, there were either one or three bases common to both

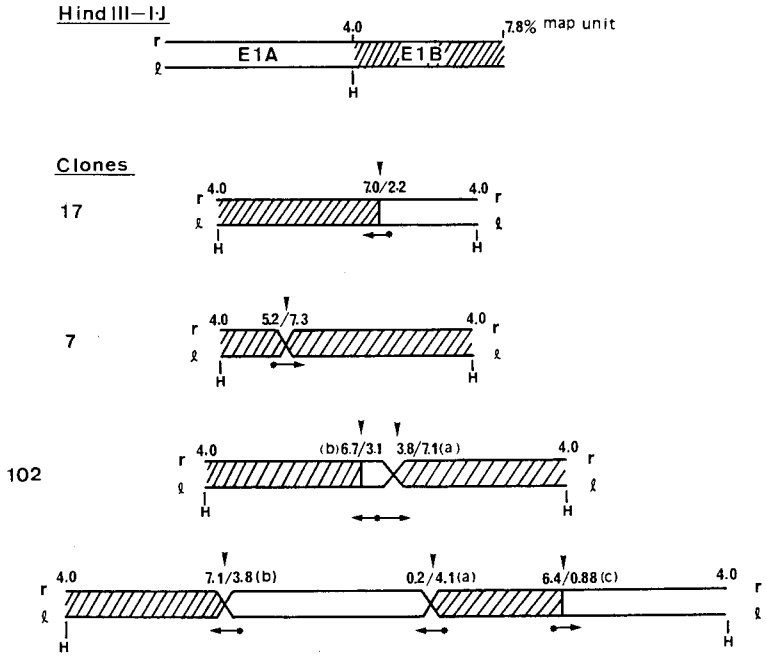

Fig. 4 Structure of the viral DNA cloned from the 7IJY1-1 cell DNA. The bars with numbers in Ad7 genome position (%) represent the Ad7 *Hin*dIII-I·J fragment (*Hin*dIII-I·J) and the cloned viral DNA (clones 17, 7, 102 and 370). Open and hatched bars represent the E1A and E1B regions respectively. Rightward (r) and leftward (l) transcription strands and the *Hin*dIII cleavage site (H) are shown. The functions are marked by arrowheads. Arrows and dots denote the extents and 5'end -labeled positions of sequence determination, respctively: *Sau*3A site (nucleotide 1704) in clone 17; *Bst*NI site (nucleotide 1249) in clone 7; *Sau*96I site (nucleotide 1249) in clone 102; *Hha*I, *Bgl*II, and *Acc*I sites (nucleotides 1314, 1540, 2178) in clone 370.

parental DNA molecules (a A in the junction 7, 370 (a), and 102 (b); a T in the junction 370 (b) and 102 (a); a GAG in the junction 370 (c); a GAA in the junction 17, Fig. 5). The junctions of 370 (b) and 102 (a) were the same.

Structure of the cloned DNAs complementary to an E1B-E1A fusion mRNA

Cytoplasmic poly A RNA ranging from 1.5 to 2.2 kb in size, previously assayed for the presence of unusual viral mRNAs by Northern blot hybridization (5), were isolated from the 7IJY1-1 cell line and converted to cDNA. The cDNA was inserted into the *Pst*I cleavage site of pBR322 DNA by homopolymeric tailings with dG and dC. Approximately, 3,000 tetracycline-resistant colonies were screened for the presence of viral DNA sequences by the colony hybridization method (14), using Ad7 [^{32}P] *Sal*I-C (0-17.5%) as a probe. A total of 5 clones that hybridized with the probe were selected (Fig. 6A) and were analyzed by restriction endonuclease

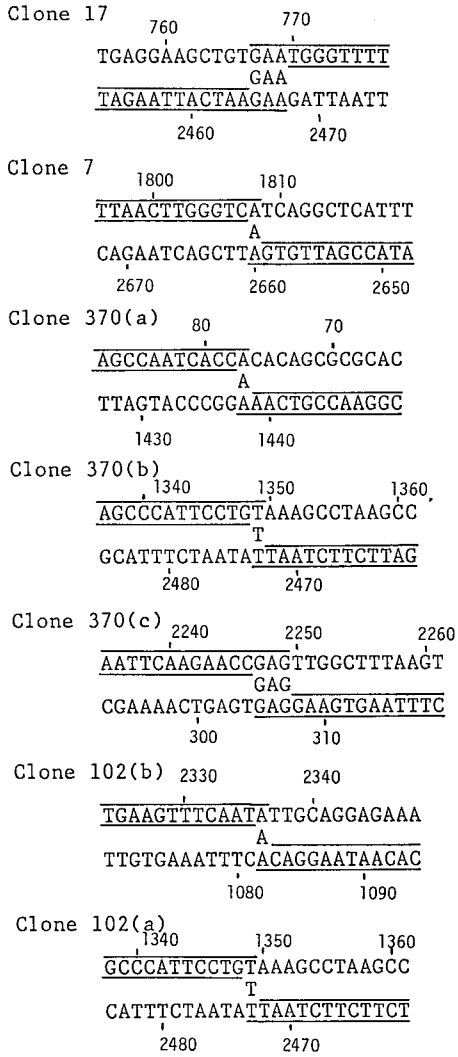

Fig. 5 Sequences at the seven virus–virus junctions of the integrated *Hin*dIII-I•J. Sequences of the junctions in clones 17, 7, 370, and 102 are presented, together with subsets of parental sequences (23–27 bases) spanning the junctions. Jointed sequences are included between two horizontal lines, and sequences outside the lines are the immediately adjacent viral sequences which have been deleted. Arrowheads represent the junctions and one or three bases common to the two joining parental DNA sequences. The numbers are given in nucleotides counted from the right-hand end of the viral genome of the Ad7 Grider strain (48).

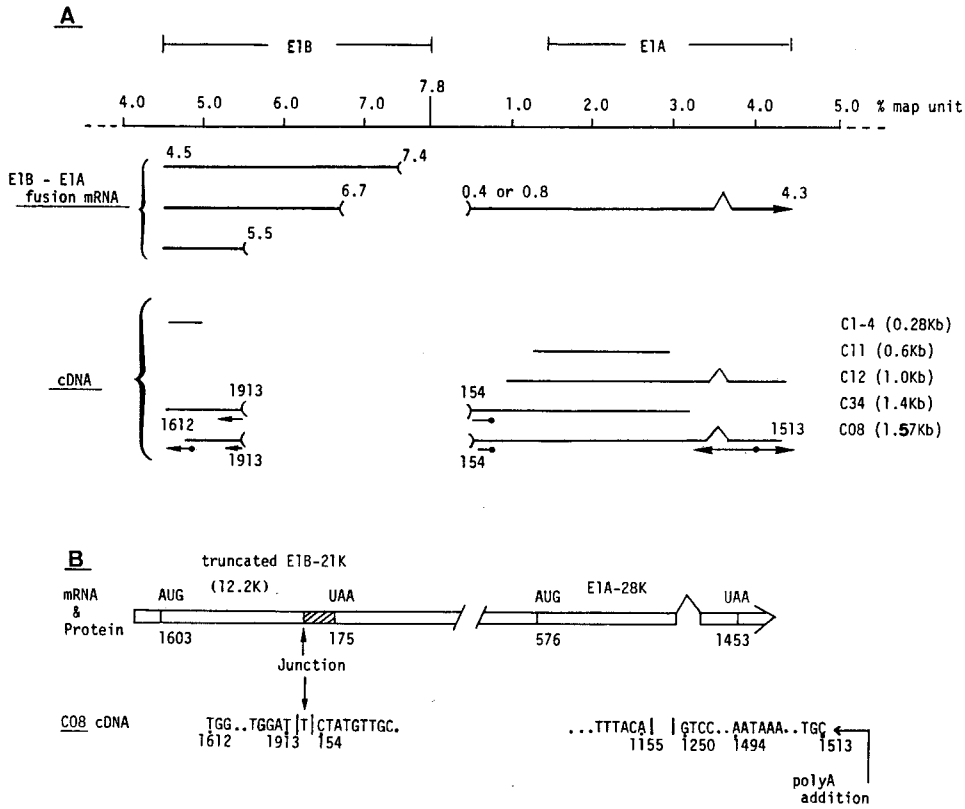

Fig. 6. Structure of E1B-E1A fusion mRNAs unique to 7IJY1-1 cells. A: Possible structure of E1B-E1A fusion mRNAs, deduced from RNA mapping results 8459 are shown by lines. The numbers above lines denote genome positions 859 of the 5' end, 3' end (arrowhead) and junction sites (parentheses) of E1B-E1A mRNAs. Carets indicate regions spliced out from the RNAs. Structure of the five cDNA clones are shown by lines. The genome positions are marked by nucleotide numbers counted from the right-hand end of the genome of Ad7 Grider strain (unpublished data). Dots (*Hae*III site; nucleotide 1689, *Acc*I site; nucleotide 215, and *Hin*dIII site; nucleotide 1384) denote the 5' end-labeled sites. Arrows show the extents of sequence determinations. B: Structure of the E1B -E1A fusion mRNA deduced from analysis of the cDNA clone, C08. DNA seguences around the 5' and 3' ends, junction, and RNA splice site of the E1B-E1A mRNAs are indicated and marked by nucleotide numbers. Bars denote the polypeptides predicted from DNA sequences. A hatched bar shows 7 amino acids continued with carboxyl -terminal of the truncated E1B-21 K protein.

digestions and Southern blot hybridization (data not shown). The result showed that the cDNA of 1.57 and 1.4 kb, referred to as C08 and C34, respectively, consist of sequences from both regions of the E1A and E1B regions (Fig. 6A). Next, we have determined part of nucleotide sequences of the cDNAs in C08 and C34 (Fig.

6B). The sequence strategy in shown in Figure 6A. The cDNAs from C08 and C34 clones overlap in most of the sequences and share E1B-E1A linkage site between nucleotide 1,910 and 154 with an insertion of a nucleotide T at the junction. The junction site is very close to the E1B-E1A junction (5.5/0.8%) of an unusual viral mRNA, which was mapped by the nuclease S1-mapping technique (5). The cDNA sequences from the C08 clone also indicated that this unusual viral mRNA has a splice donor at nucleotide 1,155, the acceptor at nucleotide 1,248, and the polyadenylation site at nucleotide 1,514. These genome positions are the same as those of splicing and polyadenylation for the 1.1 kb E1A mRNA. The cDNA sequences could not comprise the 5'end of unusual viral mRNAs, since it was lost, probably when double-stranded cDNA was digested with S1 nuclease. RNA mapping results (5), however, suggested that the unusual viral mRNAs have the same 5'end around map position 4.5% as these of the E1B mRNAs. Thus, the structure of the cloned cDNAs confirmed our previous proposal (5) in which that transcription of E1B-E1A mRNAs initiates at the E1B promoter, proceeds through the E1B sequences to the E1A sequences and terminates in the same manner as the E1A mRNAs. The cloned cDNA corresponds in size to the E1B-E1A fusion mRNA with an approximate length of 1.8 kb. Inspection of the cDNA sequences indicated that this E1B-E1A fusion mRNA theoretically coded for a carboxyl-terminal truncated form(12.2 K) of the E1B-gene-encoded 21 K protein (E1B-21 K encoded amino acid plus flanking viral DNA-encoded 7 amino acids, Fig. 6B). If the second initiation codon (ATG) at nucleotide 575 is used, it is possible that the E1A-28 K protein can be translated (Fig. 6B).

A possible DNA template for the transcription of the E1B-E1A fusion mRNA

In clone 370, the DNA (4.1-6.8%) spanning the 5'end portion of the E1B region was fused to the DNA (0.88-4.3%) spanning the E1A region at the junction at 6.8 and 0.88% (Fig. 4). This E1B-E1A fusion DNA is comparable to that of a DNA template for the transcription of one species of the E1B-E1A fusion mRNAs, as shown in figure 7. The E1B-E1A junction is very close to the E1B-E1A junction (6.7/0.8%) of one species of E1B-E1A fusion mRNAs (Fig. 6A), which was mapped by the nuclease S1-mapping technique (5). The E1B-E1A fusion DNA in clone 370 comprises a potential 5' end (4.5%) and a splice point (donor site: 3.3%, acceptor site: 3.5%)for the E1B-E1A fusion mRNAs. The 3' end of the E1B-E1A fusion mRNAs has been mapped at 4.3% outside the *Hin*dIII cleavage site (4.0%) and therefore was not included in the clone 370 DNA. These results show that the E1B-E1A fusion DNA in 370 clone serves as a template for the transcription of the E1B-E1A fusion mRNA with an approximate length of 2.0 kb. Inspection of the E1B-E1A junction sequences, together with the sequences of the *Hin*dIII-I•J (4),

revealed potential coding capacity for three kinds of proteins (Fig. 7) : a E1B-21 K protein, a carboxyl-terminal truncated polypeptide (12.7 K) of the E1B-55 K protein (but 4 amino acids continuous with carboxyl-terminal), and a E1A-28 K protein. It is unlikely that other fusion DNAs cloned are candidates for the transcription templates of E1B-E1A fusion mRNAs.

In vitro translation of hybridization-selected viral RNAs

The proteins synthesized *in vitro* in the messenger RNA-dependent rabbit -reticulocyte-lysate-system using virus-specific RNAs from Ad 7-infected KB cells and the transformed 7IJY1-1 line are shown in figure 8. RNAs encoded in the E1A region and the E1B region were selected by hybridization with the DNA fragments spanning 0 to 4.0% (for E1A) and 4.5 to 7.8%, 5.5 to 8.3%, and 8.3 to 10.2% (for E1B). Translation of E1A-selected RNAs isolated at 6 h post infection from Ad 7 -infected KB cells resulted in the synthesis of the proteins with apparent molecular weights of 45,000 and 34,000 (E1A-45 K and E1A-34 K ; lanes 4 and 5). These proteins must be related to the E1A-28 K and E1A-24 K predicted in the E1A region from DNA sequence analysis (4, 6).Translation of E1B-selected RNAs resulted in the synthesis of two major proteins of 58,000 and 16,000 (E1B-58 K, E1B-16 K, ; lane 2). These probably correspond to the E1B-55 K and E1B-21 K proteins predicted in the E1B region from DNA sequence analysis (4). E1B-selected RNAs isolated at 20 h post infection produced as additional protein of 12,000, possibly corresponding to the virion component protein of polypeptide IX (lanes 4 and 8).

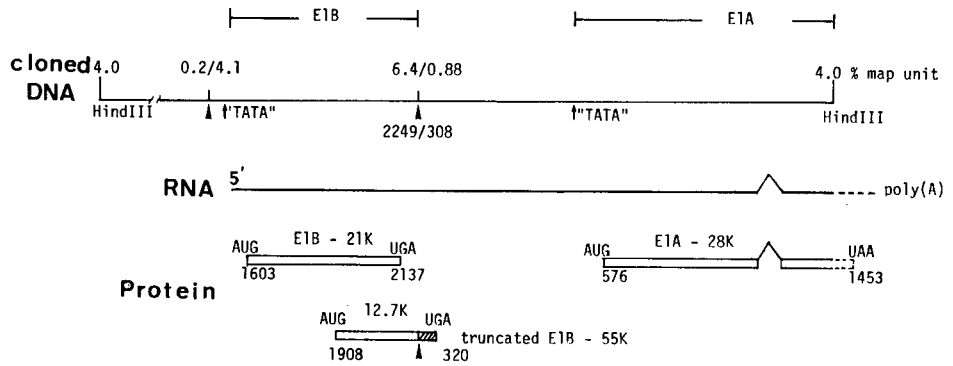

Fig. 7 A possible DNA template for transcription of the E1B-E1A fusion mRNA. The DNA (4.2 kb) cloned in 370 from the 7IJY1-1 cell DNA are indicated and marked off with Ad7 genome position (%). Arrowheads denote junction sites. The E1B-E1A fusion mRNA and its coding potential are shown by line and bars, respectively. A hatched bar denotes 4 amino acids continued with carboxyl-terminal of the truncated E1B-55 K. The 3' -terminal portion of mRNA and carboxyl-terminal portion of the E1A-28 K protein (interrupted lines) are not included in the clone 370 DNA.

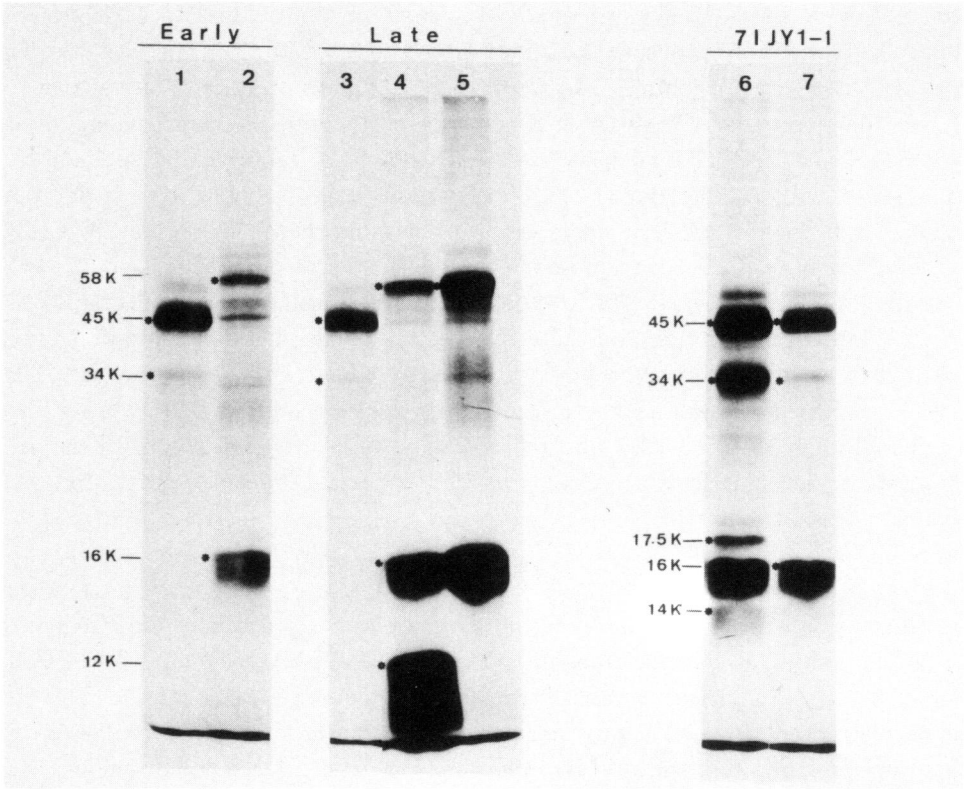

Fig. 8 SDS-polyacrylamide gel electrophoresis of *in vitro* translation products of hybridization-selected viral mRNAs from Ad7-infected KB cells and 7IJY1-1 cells. Shown in a fluorography of 11% polyacrylamide-SDS gels of [^{3}H] leucin-labeled *in vitro* translation products made by using viral mRNAs selected by hybridization with E1A probe (*Hin*dIII-I; 0-0.4%, lanes 3 and 6) and E1B probe(*Hin*dIII-J/*Bgl*II; 4.5-7.8%, lanes 2 and 7, *Bam*HI-G; 5.5-8.3%, lane 5, *Bam*HI-I; 8.3-10.2%, lane 4). RNAs for hybridization-selection were isolated from Ad7-infected KB cells at an early stage (6 h-postinfection, lanes 1 and 2) and at a late stage (20 h-postinfection, lanes 3, 4, and 5) during infection and from 7IJY1-1 cells (lanes 6 and 7). The apparent molecular weights of proteins were calculated by relative mobilities to marker proteins (lysozyme; 14,000, soybean trypsin inhibitor; 21,500, carbonic anhydrase; 31,000, ovalbumin; 45,000, bovine serum albumin; 66,200, phosphorylaseB; 92,500, and β-galactosidase; 116,250.)

Next, we compared these proteins with those made using viral RNAs prepared from 7IJY1-1 cells. Translation of these mRNAs yielded three major proteins with 45 K, 34 K, 16 K, and two minor proteins with 17.5 K and 14 K (lanes 6 and 7). Proteins of 45 K,34 K, and 16 K must be identical to the E1A-45 K and E1A-34 K proteins and the E1B-16 K proteins, respectively, which were produced from RNAs selected from Ad7-infected KB cells. In the 7IJY1-1 cell line, however, large amounts of

the E1A-45 K and E1B-16 K proteins and a small amount of the E1A-34 K protein could be translated from both species of RNAs selected by E1A and E1B-specific probes. E1B-E1A fusion mRNAs can be fished by both probes and are shown to have coding potential of the E1A-45 K and E1B-16 K (proteins corresponding to the E1A-28 K and E1B-21 K proteins predicted from DNA sequences)(Fig. 6 and 7). Therefore, we concluded that the E1A-45 K and E1B-16 K proteins were made from E1B-E1A fusion mRNAs. In addition, it appears that the E1A-45 K and E1A-34 K proteins are also translated from the 1.1 kb and 0.9 kb E1A mRNAs present in 7IJY1 -1 cells. A minor protein of 14 K was detected in the translation products of mRNAs from 7IJY1-1 cells but not from Ad 7-infected KB cells. The 14 K protein may be related to the truncated polypeptide of the E1B-16 K or E1B-58 K protein. The 17.5 K protein could be detected also in the translation products directed by E1B -selected mRNAs from Ad 7-infected KB cells for a longer exposure of autoradiography (data not shown). Coding position at the viral genome for this protein is unknown.

DISCUSSION

In this paper, we have analyzed the structure and expression of the integrated viral DNA in a rat cell line transformed by the *Hin*dIII-I•J (0-7.8%) of the Ad7 DNA. Integration of adenovirus DNA or DNA fragments into the host cell DNA is a primary event for cell transformation by adenovirus or adenovirus DNA. It appears that the site of viral DNA integration in the cell DNA in not unique, and viral sequences involved in the integration are not limited to a specific site of the viral genome (reviewed by Doerfler ; ref. 23). Comparison of the DNA before and after integration into the host cell DNA indicated that parental DNA molecules were often altered and joined together, including deletions, duplications, base substitutions, and insertion of host DNA (24-27). Some of the cell lines transformed by adenovirus, adenovirus DNA, and its fragments exhibited tandem integration patterns of viral DNAs (28-30). In the 7IJY1-1 cell line, the transfected *Hin*dIII-I•J were integrated at the two fragments of the *Eco*RI-cleaved cell DNA and joined together at various sites in a head to tail or a head to head fashion. At all the seven junctions examined, one or three bases were found to be shared between the two joining parental sequences. Such a common base has been reported in other systems: (1) two of the six viral-viral DNA iunctions in natural arising SV40 variants (31) ; (2) three of the six junctions of viral DNAs in circulization of transfecting linearized SV40 DNA (32) ; (3) five or three base homology shared by SV40 and cellular sequences at the cell-virus junctions of SV40-transformed rat SVRE9 cells (33) and 14 B cell (34) ; (4) one or two base homology at the virus -virus DNA junctions of adenovirus 12 mutants which were adapted to growth in

human tumor lines (35). It is unclear whether a short homology at the junction point plays a role in the linkage of DNAs. The richness of A : T base pair or patch -like homology in parental sequences spanning the junction has been reported (23, 31, 32). Here, some of the viral sequences were A+T rich (76% A+T in the junctions of 102 (b) and 370 (b), 78% A+T in 17 junction) or exhibited a patch -like homology (102 (a) junction), but others did not.

Expression of viral DNAs in the transformed cell is often complicated by altered integration structure of viral DNA templates. Primarily, Sambrook *et al* (28) showed that according to the integration structure of viral DNA templates, certain transformed cell lines would synthesize new mRNAs different from authentic viral mRNAs produced in productive cycle of virus propagation. The 7IJY1-1 cell line and its analogous 7IJY1-2 and 7IJY1-3 cell lines, produced two or three species of unusual viral mRNAs, in addition to the same species as the two E1A mRNAs produced in Ad 7-infected KB cells (5). In this study, we confirmed that unusual viral mRNAs were E1B-E1A fusion mRNAs and that their transcription initiated at or near the E1B promoter, proceeded through the E1B sequences into the flanking E1A sequences, and terminated in the same manner as the E1A mRNAs. Although the poly A addition signal for the E1B mRNAs is lost in the *Hin*dIII-I•J, it was substituted by that for the E1A mRNAs at the flanking E1A sequences. In a polyoma tsA-transformed mouse cell line, the viral DNA sequences containing the poly A addition site for early mRNA has been deleted and substituted by that at flanking mouse DNA (36). In a adenovirus 2-transformed rat F4 cell line, the termination signal for the E4 mRNAs has been lost and replaced by that for E1A mRNAs, resulting in the transcription of E4-E1A fusion mRNAs (28). Such a chimeric type of mRNAs was also observed in rat cell lines transformed by the Ad12 *Hin*dIII-G (0-6.8%) and the polyoma virus (37, 38).

Comparison of rat cells transformed by the left-terminal 4.5% and 6-8% fragments of adenovirus DNA suggests that gene products specified by the DNA region between 4.5-8% an responsible for the establishment of completely transformed cells carrying phenotype characteristic of whole viral DNA-transformed cells. The region of adenovirus 5, 7, and 12 DNA between 4.5% and 8% specifies the E1B gene-encoded protein of 19,000~21,000 (E1B-19 K~21 K) and amino-terminal portion of the E1B gene-encoded 55,000 protein (E1B-55 K) (39). Transformation of rat cells by the E1B-19 K protein-deficient mutants of Ad2, Ad5, and Ad12 indicated that lesion of the E1B-19 K protein resulted in the reduction or abolishment of focus formation or soft agar colony formation (40-45). Since the 7IJY1 -1 cell line was isolated from cell colony grown in soft agar, it seems that the expression of the Ad7 E1B-21 K protein, related to the Ad5 and Ad12 E1B-19 K (39) , must be under selection pressure. Tandem integration of the *Hin*dIII-I•J made it

possible to transcribe E1B-E1A fusion mRNAs and to synthesize the E1B-21 K protein (apparent molecular weight of 16,000 in SDS-polyacrylamide gels). In contrast, baby rat kidney cells transformed by the Ad12 *Hin*dIII-G (0-6.8%) and rat 3Y1 cells transformed by the Ad31 *Hin*dIII-G (0-6.7%) exhibited incompletely transformed phenotypes, reducing or lacking growth in soft agar and in tumorgenicity in transplanted newborn rats or nude mice (46, 47). However, these cell lines were selected under less stringent conditions (focus formation in liquid culture medium), and cell lines examined were shown to express the E1B gene in much lower abundance or at an undetectable level (46, 47). Therefore, it will be reasonable to consider that the left-terminal 7-8% fragment of the viral DNA can induce soft agar growing and tumorgenic transformed cells, although the enhanced expression of the E1B gene product (probably the Ad7 E1B-21 K or its analogues of Ad5 and Ad12) is required. Recently, Shiroki *et al.* (22) showed by cotransfection experiments that expression of the E4 gene is required for the establishment of the Ad 12 E1-transformed rat 3Y1 cell lines which are capable of growing in soft agar. They suggested that the E4 gene contributes indirectly to the establishment of soft agar growing transformed cells through the enhancement of the E1B gene expression. The 7IJY1-1 cells examined include no sequences derived from the E4 gene. Our data indicated that the expression of the E4 gene is not absolutely required for colony formation of transformed cells in soft agar. Babiss *et al.* (40) has isolated a series of Ad5 mutants that contain alteration in the E1B-55 K protein, and these were shown to be reduced in growth rate in soft agar, in relation to the size of the truncated polypeptides. It seems that the contribution of truncated polypeptides of the E1B-55 K protein to transformation is also considered.

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