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Isolation of a Nondefective Recombinant Between Adenovirus Type 5 and Early Region 1A of Adenovirus Type 12

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A nondefective recombinant between adenovirus type 5 (Ad5) and type 12 (Ad12), rc-1 (Ad5 *dl*312, carrying the Ad12 E1A gene), was isolated from hamster cell foci transformed by a defective recombinant, rcB-1 (*dl*312, carrying the Ad12 E1 gene). The recombinant rc-1 grew in human embryo kidney and KB cells in the absence of helper and synthesized Ad12 T antigen g, the product of the E1A gene. The genome of rc-1 has a deletion between 79.9 and 82.5 map units of Ad5 *dl*312 DNA with an insertion of 0.1 to 5.5 map units of Ad12 DNA at the deletion site. The mRNAs of Ad12 E1A were transcribed from the Ad12 E1A promoter, and unusual RNAs were abundantly transcribed from the Ad5 E3 promoter on the opposite strand. The frequency of cell transformation with rc-1 was lower than those with Ad5 and Ad12 wild types.

Adenovirus type 12 (Ad12) is highly tumorigenic in newborn hamsters (19) and is able to transform rat, hamster, and mouse cells in culture. Ad12-transformed cells form tumors efficiently when transplanted into syngeneic animals (5). Both Ad2 and Ad5 are non-tumorigenic in newborn hamsters but are able to transform rat cells in culture. However, Ad2- or Ad5-transformed cells form tumors only inefficiently or form no tumor when transplanted into animals (6, 15). It is of interest to know the difference in the efficiency for tumor formation between Ad12 and Ad5. To this end, we have isolated recombinants between a deletion mutant of Ad5 (*dl*312) deleted in the Ad5 E1A gene (8) and Ad12 DNA fragments containing the Ad12 E1 gene (15). Rat cells were transformed with the recombinants, and these transformed cells were transplanted into rats to determine the efficiency of tumor formation. The results show that efficient tumor formation is dependent on expression of the Ad12 E1B gene. However, these recombinants are defective and grow in KB and human embryo kidney (HEK) cells with complementation of Ad5 *dl*312. This paper describes the isolation of a nondefective recombinant, rc-1, from the defective recombinant, rcB-1, and transformation of cells with rc-1.

Hamster cells were infected with the defective recombinants rcB-1 and rcB-2 (15) and cultured at 37°C in a CO_2 incubator. Several foci of transformed cells appeared 3 weeks after infection. Concomitantly, cell degeneration became apparent in some cultures in which transformed foci were detected. When cell medium lysates of cultures with such foci were plaque assayed in HEK cells, several plaques were formed with lysate from the culture infected by rcB-1, and a clone of the recombinant, rc-1, was obtained from the plaque. The recombinant, rc-1, grew well in HEK, KB, and 293 (7) cells at one-hit kinetics and grew slightly in hamster cells. However, it did not grow well in rat 3Y1 cells (9) (Table 1). The recombinant, rc-1, formed Ad12 T antigen g, the product of the Ad12 E1A gene, in infected cells, as determined by immunofluorescence (Table 1) and two-dimensional gel electrophoresis after immunoprecipitation (data not shown) (14, 17).

The genome structure of rc-1 was analyzed by restriction mapping followed by Southern blot hybridization (11, 15, 18). The analysis of Ad12 mRNA in rc-1-infected cells indicates that the Ad12 sequence from nucleotide position 50 to position 1950 is integrated in rc-1 DNA, as shown below. Assuming that this notion is correct, the analysis was carried out under the assumption that the fragment of Ad12 DNA inserted in rc-1 DNA is about 1,900 base pairs, from 0.1 to 5.5 map units (m.u.) of Ad12 DNA. Size (m.u.) of Ad12 DNA is converted to percent in terms of Ad5 DNA as 100%. The positions of insertion and deletion in rc-1 were analyzed by the sizes of fragments generated by cleavage with restriction enzymes *Xho*I and *Kpn*I and by the sizes of fragments hybridized with ^{32}P-labeled Ad12 DNA (Fig. 1). In rc-1 DNA, the *Xho*I-D fragment (14.0%) is missing. Since the rc-1 fragment B appears more dense

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TABLE 1. Virus propagation and formation of Ad12 T antigen g in infected cells

Virus	Titer (PFU/0.2 ml)[a]		Growth (PFU/0.2 ml)[b]		Ad12[c] g
	293	HEK	Hamster	Rat 3Y1	
rc-1	$10^{7.5}$	$10^{8.3}$	10^6	5×10^2	+
rcB-1	$10^{8.5}$	$10^{7.0}$	10	ND	+
*dl*312	$10^{8.5}$	$10^{4.0}$	$\leqq 10$	$\leqq 10^3$	−
Ad5	$10^{8.5}$	$10^{9.0}$	10^7	10^3	−
Ad12	$10^{6.5}$	$10^{7.2}$	$\leqq 10$	$\leqq 10^2$	+

[a] The virus was propagated in KB, HEK, or 293 cells (for *dl*312). The virus titers were plaque-assayed in 293 or HEK cells, and each titer is shown in PFU per 0.2 ml.

[b] Hamster or rat 3Y1 cells were infected at 10 PFU/cell, and the cell-medium lysate at 48 h after infection was plaque-assayed in HEK or 293 cells (for *dl*312). Each titer is shown in PFU per 0.2 ml.

[c] HEK cells were infected on a cover slip with the virus, and T antigen g was examined by immunofluorescence. +, T antigen g positive; − negative.

than the *dl*312 DNA fragment B in the gels stained with ethidium bromide (Fig. 1), an additional fragment would seem to comigrate with the rc-1 fragment (16.8%). Southern blot analysis indicates that the comigrated band contains the Ad12 DNA sequence. From this we conclude that the Ad12 DNA sequence is inserted in the *Xho*I-D fragment of Ad5 DNA and that rc-1 DNA is longer than *dl*312 DNA by 2.8% (16.8% minus 14.0%). As described above, the size of Ad12 DNA inserted in rc-1 DNA is 5.4%. Therefore, rc-1 DNA has a deletion (2.6%, or 5.4% minus 2.8%) of Ad5 DNA in the *Xho*I-D fragment. *Kpn*I-D (12.7%) and -F (8.9%) fragments are missing in rc-1 DNA, and two additional fragments (12.4 and 11.9%) are detected. The difference in size between the missing and the newly detected fragments is 2.8%, in agreement with the above results. After hybridization with ^{32}P-labeled Ad12 DNA, both additional bands are radioactive, and the radioactivity of the smaller fragment (11.9%) is higher than that of the larger one (12.5%). Since *Kpn*I cleaves Ad12 DNA at map unit 1.6, the shorter fragment contains the larger portion of Ad12 DNA and the longer fragment contains the smaller portion of Ad12 DNA; specifically, the smaller fragment (11.9%) contains 3.9% of Ad12 DNA (1.6 to 5.5 m.u.) and 8.0% of Ad5 DNA. The longer fragment (12.5%) contains 1.5% of Ad12 DNA (0.1 to 1.6 m.u.) and 11.0% of Ad5 DNA. Since the *Kpn*I-F fragment (8.9%) of Ad5 DNA is smaller than 11.0%, the 11.0% portion of Ad5 DNA must originate from the *Kpn*I-D fragment (12.7%), whereas the 8.0% portion of Ad5 DNA originates from the *Kpn*I-F fragment. This indicates that Ad12 DNA is inserted in rc-1 DNA in the reverse orientation. The left endpoint of the deletion is located 8.0% right of the left end of the *Kpn*I-F fragment (71.9 m.u.), that is, at 79.9 m.u. The right endpoint of the deletion is located 11.0% left of the right end of the *Kpn*I-D fragment (93.5 m.u.), that is, at 82.5 m.u. As shown in the diagram at the bottom of Fig. 1, rc-1 DNA has a deletion (2.6%) of Ad5 DNA between 79.9 and 82.5 m.u. and an insertion (5.4%) of Ad12 DNA at the deletion site in the reverse orientation.

When transformation assays were carried out in rat 3Y1 cells, transformed foci appeared in cells infected at 10 PFU/cell with the defective recombinant rcB-1 or with Ad12 or Ad5 wild type (WT), whereas no transformed foci appeared in cells infected with the nondefective recombinant, rc-1, or with *dl*312 during the 5-week observation period. The rc-1 recombinant did not grow in 3Y1 cells, but did show strong cell degeneration in these same cells. Since UV irradiation of Ad5 is known to reduce cell degeneration, a transformation assay was carried out with UV-irradiated virus. UV-irradiated rc-1 induced several foci after a prolonged incubation of 8 weeks (Table 2, experiment 1). Cell clones transformed with rc-1 were established from the foci. In repetitive experiments, only tiny transformed foci discernible after staining with Giemsa were detected in 3Y1 cells infected with 10^6 PFU of rc-1 (Table 2, experiment 2). The results indicate that the nondefective recombinant, rc-1, is less efficient in transformation of cells than the defective recombinant rcB-1, Ad5 WT, and Ad12 WT.

To determine the mRNA species transcribed from the inserted Ad12 region 1 DNA sequence, cytoplasmic RNAs from rc-1-infected KB cells and rc-1-transformed rat cells were hybridized with the uniformly ^{32}P-labeled Ad12 *Eco*RI-C fragment (0 to 16.5 m.u.), digested with nuclease S1, and electrophoresed on neutral and alkaline agarose gels (Fig. 2A) (1). Three bands of 620, 530, and 310 nucleotides long were observed in the alkaline gel, indicating that the major Ad12 E1A mRNA species, 1A-1 (620 + 310 nucleotides) and 1A-2 (530 + 310 nucleotides) (13), are present in rc-1-infected and -transformed cells. Judging from the intensity of these bands, less Ad12 mRNA is transcribed in rc-1-infected cells than in rcB-1-infected and rc-1-transformed cells. Three mRNA species, corresponding to prominent bands of 1,900, 1,700, and 400 nucleotides on both neutral and alkaline gels, were detected in rc-1-infected cells, showing that these species have no spliced structure within the inserted Ad12 DNA sequence. These three species were observed neither in rcB-1-infected cells nor in Ad12-infected and -transformed cells.

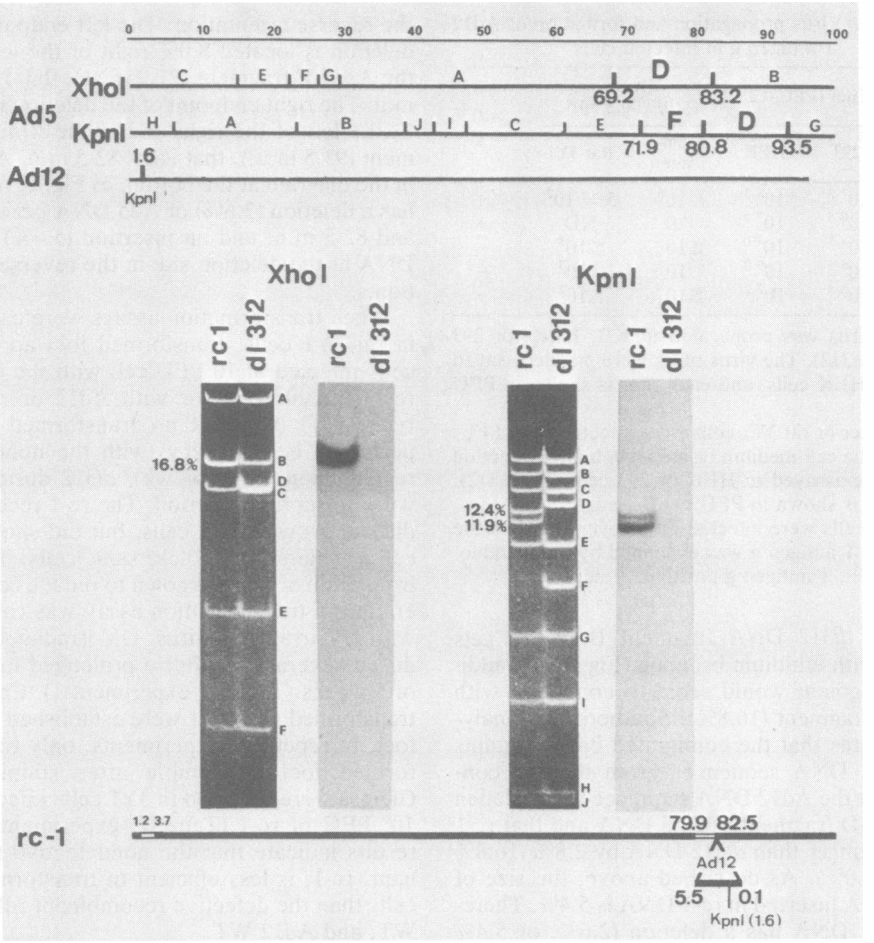

FIG. 1. Size analysis of rc-1 and *dl*312 DNA fragments hybridized with Ad12 DNA. DNAs from rc-1 and *dl*312 were digested with restriction endonuclease *Xho*I and *Kpn*I and separated by electrophoresis in 1% agarose gels. The DNA bands were visualized by staining with ethidium bromide (left columns). The bands in the gels were transferred to nitrocellulose filters by blotting and hybridized with ^{32}P-labeled Ad12 DNA. Autoradiograms of hybridized bands are shown (right columns). Restriction endonuclease cleavage maps of Ad5 DNA are shown above the gels. The structure of rc-1 DNA is shown below the gels.

To determine the transcriptional direction of the species of 1,900 and 1,700 nucleotides, recombinant pAE1A DNA (pBR322 containing Ad12 DNA from the left terminus to the *Acc*I site at position 1595) (16) was cleaved at the Ad12-pBR322 junction with *Acc*I. Ad12 fragments were terminally labeled with ^{32}P at the 5′ end with T4 polynucleotide kinase or at the 3′ end with T4 DNA polymerase and used as probes. The labeled probes were hybridized with cytoplasmic RNA from rc-1-infected cells. The hybrids were digested with nuclease S1 and analyzed on alkaline gels. Both of the *Acc*I ends were terminally labeled, but only the label at the Ad12 *Acc*I end (position 1595) could be protected from S1 digestion. The pair of bands of 1,550 and 1,350 nucleotides were detected again with the 3′-labeled probe, but no band was observed with the 5′-labeled probe (data not shown). The result indicates that the pair of species are transcribed in the direction opposite to that of Ad12 E1A mRNAs. Considering the direction of the integrated Ad12 DNA sequence, the mRNA species 1A-1 and 1A-2 are transcribed from right to left, whereas the mRNA species of 1,900 and 1,700 nucleotides are transcribed from left to right, in the same direction as Ad5 E3 mRNAs. These results indicate that the mRNA species 1A-1 and 1A-2 are transcribed from the Ad12 E1A promoter, whereas the unusual mRNA species of 1,900 and 1,700 nucleotides are transcribed from the Ad5 E3 promoter on the opposite strand.

To investigate the mRNA species from Ad5

TABLE 2. Transformation of rat 3Y1 cells

Virus	No. of foci per dish[a]							
	Expt 1[b]			Expt 2		Expt 3		
	5×10^6, 5 wks	UV, 5×10^6		10^6	10^7	10^4	10^5	10^6
		5 wks	8 wks					
rc-1	0	0	10	1.5	0	0	0	0
rcB-1	3	42	50	ND	ND	ND	ND	ND
Ad5 WT	1.5	1	10	145	81	0.2	1.2	26
Ad12 WT	30	60	70	391	TNTC	0.6	4.8	75
*dl*312	0	0	0	ND	ND	ND	ND	ND

[a] Rat 3Y1 cells (10^6 cells per dish) were infected with the indicated virus with the titer (PFU) shown in the table. After cultivation for 5 weeks, the number of foci in each was calculated, and the average number of foci in four to five dishes is shown. ND, Not done; TNTC, too numerous to count.

[b] Rat 3Y1 cells (10^6 cells per dish) were infected with the indicated unirradiated or UV-irradiated viruses at 5×10^6 PFU per dish. For UV-irradiated viruses, the results after cultivation for 8 weeks are also shown.

DNA, RNAs from rc-1-infected and -transformed cells were analyzed with uniformly labeled Ad5 whole DNA (Fig. 2B). The bands derived from the E3 region of Ad5 *dl*312-infected cells (Fig. 2B, lane 3) disappeared in rc-1-infected and -transformed cells (Fig. 2B, lanes 4 and 5). These results are consistent with the substitution of rc-1 DNA in the E3 region.

The analysis of the viral genome in a nondefective recombinant, rc-1, revealed that rc-1 DNA had deletions in the regions for Ad5 E1A and E3 and an insertion of Ad12 E1A at the deletion site for Ad5 E3. Since a portion (80 to 86 m.u.) of the Ad2 E3 gene (76 to 86 m.u.) is deleted in nondefective Ad2-SV40 hybrid virus, it is believed that the region of the Ad2 E3 gene is not indispensable for virus growth (10). It has been shown that the Ad12 E1A gene is able to

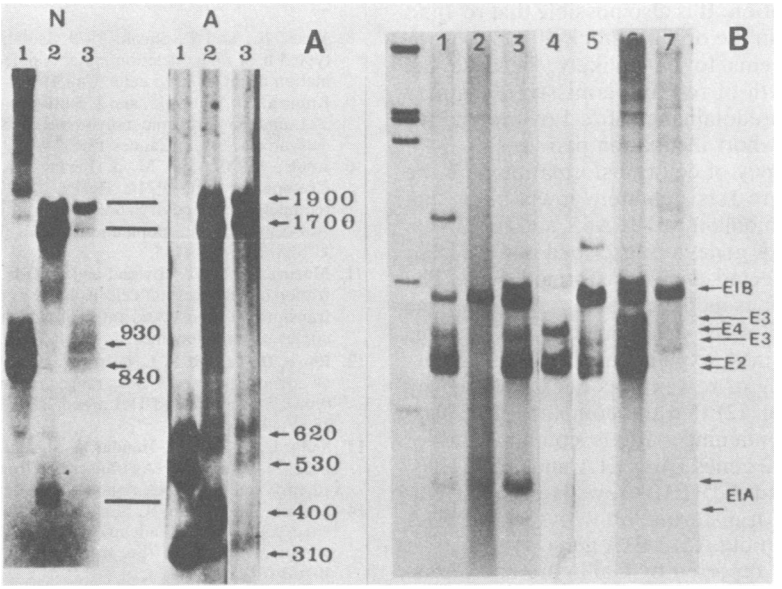

FIG. 2. (A) Autoradiograms of nuclease S1-digested RNA-DNA hybrids between cytoplasmic RNA and the Ad12 *Eco*RI-C fragment (0 to 16.5 m.u.). Cytoplasmic RNAs were extracted from KB cells infected with rcB-1 (lane 1) and rc-1 (lane 2) and from cells transformed with rc-1 (lane 3), hybridized with the ^{32}P-labeled Ad12 probe, digested with nuclease S1, and analyzed by electrophoresis in neutral (N) and alkaline (A) gels. (B) Autoradiograms of S1-digested RNA-DNA hybrids between Ad5 DNA and cytoplasmic RNA. Cytoplasmic RNAs were prepared from KB cells infected with Ad5 (lane 1), rc-1 (lane 4), or rcB-1 (lane 6), from 3Y1 cells transformed with Ad5 (lane 2), rc-1 (lane 5), or rcB-1 (lane 7), and from 293 cells infected with *dl*312 (lane 3). The *Xho*I digest of Ad5 DNA is shown at left as a size marker.

complement the Ad5 E1A gene (3, 12, 15, 20). The defective rcB-1 DNA has a deletion between 71 and 82 m.u., whereas the nondefective rc-1 DNA has a deletion between 79.9 and 82.5 m.u. Thus, the region between 71 and 80 m.u., which encodes L4 proteins (4), is deleted in rcB-1 DNA and present in rc-1 DNA. This difference may explain the defectiveness of rcB-1 and nondefectiveness of rc-1.

The most plausible explanation for the mechanism of the nondefective rc-1 formation may be recombination between *dl*312 and rcB-1 DNA. The growth of rcB-1 (mixture of defective rcB-1 and *dl*312) is inefficient in semipermissive hamster cells, resulting in the formation of transformed foci after incubation for 30 days. It is also possible that rcB-1 and *dl*312 DNAs replicate to a limited extent and have a chance to recombine in a long incubation period, resulting in formation of the nondefective rc-1 DNA, in which the right portion of rcB-1 DNA (82.5 to 100 m.u.) is recombined with the left portion of *dl*312 (0 to 79.9 m.u.), with the deletion of Ad5 DNA (79.9 to 82.5 m.u.) and the insertion of the Ad12 sequence (0.1 to 5.5 m.u.) at the deletion site. Once rc-1 DNA is formed, it may replicate more efficiently in hamster cells than the original rcB-1, resulting in the appearance of cell degeneration. The difference in the size of the deletion between rcB-1 and rc-1 may also be explained by recombination. It is also possible that rc-1 is a contaminant in the original stock of rcB-1; however, this seems to be unlikely, because the efficient growth of rc-1 in permissive cells may result in the predominance of rc-1 over defective rcB-1 after a short incubation period.

The frequency of cell transformation with the recombinant rc-1 is definitely lower than that with the recombinant rcB-1, Ad5, and Ad12 WT. The Ad12 E1A gene is transcribed and translated in rc-1-infected and -transformed cells. The transformation is induced by the Ad12 E1A gene and the Ad5 E1B gene in rc-1 DNA. This combination may explain the inefficient transforming ability of rc-1. It has been reported by Bernards et al. (2) that the comparison of plasmid DNA containing various combinations of Ad5 and Ad12 genes (Ad5 E1A and Ad12 E1B, Ad12 E1A and Ad5 E1B) reveals a higher efficiency of cell transformation with the Ad5 E1A gene than with the Ad12 E1A gene. However, it has also been reported by Gallimore and Paraskeva (6) that Ad5 and Ad12 transform rat cells with the same frequency. Thus, the reason for the lower transforming ability of rc-1 remains to be explained. Despite its lower transforming ability, rc-1 can replicate in permissive cells as efficiently as Ad5 WT. This observation suggests that the Ad12 E1A gene functions sufficiently for virus growth but insufficiently for cell transformation. The mechanism for this observation is not known.

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