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Recombination in hamster cell nuclear extracts between adenovirus type 12 DNA and two hamster preinsertion sequences

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A cell-free system of nuclear extracts from BHK21 cells has been developed to catalyse recombination *in vitro* between the DNA of adenovirus type 12 (Ad12) and two different hamster preinsertion sequences. The pBR322 cloned 1768 bp fragment p7 and the 3.1 kbp fragment p16 from BHK21 hamster DNA had previously been identified as the preinsertion sites corresponding to the junctions between Ad12 DNA and hamster DNA in cell line CLAC1 and in the Ad12-induced tumour T1111(2), respectively. Preinsertion sequences, which had recombined previously with foreign (Ad12) DNA, might again be recognized by the recombination system even in a cell-free system. *Pst*I cleaved Ad12 DNA and the circular or the *Eco*RI linearized p7 or p16 preinsertion sequences were incubated with nuclear extracts. Recombinants were isolated by transfecting the DNA into recA$^-$ *Escherichia coli* strains and by screening for Ad12 DNA-positive colonies. Without a selectable eukaryotic marker, all Ad12 DNA positive recombinants were registered. Out of a total of >90 p7-Ad12 DNA recombinants, 21 were studied by restriction-hybridization, and four by partial nucleotide sequence analyses. Among the p16-Ad12 DNA recombinants, four were analysed. The sites of linkage between Ad12 DNA and p7 or p16 hamster DNA were all different and distinct from the original CLAC1 or T1111(2) junction site between Ad12 and hamster DNA. The *in vitro* recombinants were not generated by simple end-to-end joining of the DNA fragments used in the reaction but by genetic exchange. Thirteen of the 25 recombinants were derived from the 61-71 map unit fragment of Ad12 DNA. Recombination experiments between Ad12 DNA and four randomly selected unique or repetitive hamster DNA sequences of 1.5-6.2 kbp in length did not yield recombinants. Apparently, the p7 and p16 hamster preinsertion sequences recombined with Ad12 DNA with a certain preference.

Key words: Integration of viral DNA/cell free recombination/nucleotide sequence of recombinants/preinsertion sequences

Introduction

In early research on the integration of foreign DNA into the genome of mammalian cells, BHK21 hamster cells were abortively infected with adenovirus 12 (Ad12) virions, and the intracellular fate of the viral DNA was determined. Covalent linkage between Ad12 viral DNA and hamster DNA was demonstrated (Doerfler, 1968, 1970). Later, we studied the linkage between adenovirus DNA and cellular DNA in clonal lines of adenovirus-transformed cells or of Ad12-induced tumour cells. Nine different junction sites were analysed in detail (Doerfler *et al.*, 1983, 1985, 1987; Lichtenberg *et al.*, 1987; Jessberger *et al.*, 1989). Their structures did not reveal common or specific cellular sequences at sites of recombination. Frequently, patchy homologies between viral and cellular sequences were observed at or close to the sites of junction (Deuring *et al.*, 1981; Stringer, 1981; Gahlmann *et al.*, 1982; Stabel and Doerfler, 1982; Schulz and Doerfler, 1984). Many of the cellular preinsertion sites corresponding to sites of linkage between mammalian cell and adenovirus DNA were shown to be transcriptionally active. The altered chromatin structure at the sites of transcription was thought to have enhanced the chances of recombination at these cellular DNA sequences (Gahlmann *et al.*, 1984; Lichtenberg *et al.*, 1987; Schulz *et al.*, 1987).

In the present report, a cell-free system of nuclear extracts from BHK21 hamster cells is described in which different non-homologous recombinants between Ad12 DNA and the p7 or p16 hamster cell preinsertion sequence were produced *in vitro*. Out of a total of >90 recombinants, the structures of 25 different recombinants were determined by restriction enzyme cleavage, by Southern blot hybridization and in part by nucleotide sequence analyses. Four randomly selected hamster DNA sequences, which were unrelated to the preinsertion sequences, did not give rise to recombinants with Ad12 DNA at frequencies comparable to the p7 or the p16 preinsertion sequence.

Results

Hamster preinsertion sequences as targets for Ad12 DNA recombination

The hamster preinsertion sequences, p7 or p16, were incubated *in vitro* with Ad12 DNA together with nuclear extracts from BHK21 cells, in order to test whether the hamster preinsertion sequences would also be capable of recombining with Ad12 DNA in a cell-free system. The scheme in Figure 1a presents a detailed map of the p7 construct and the nucleotide sequence of the p7 DNA (Figure 1b). The CLAC1 preinsertion site and the sites of recombination with Ad12 DNA in the *in vitro* generated recombinants p7-R5 and p7-R6 are also indicated. The p7 sequence carries GCCC repeats, a CCTCTCCG sequence occurring at or close to two recombination sites and noticeable CCTT and CTG repeats (Figure 1c and d), as well as TGG repeats and other sequences designated. Multiple stem-loops concentrated towards the right end of the sequence are also indicated. The nucleotide sequence in p7 has several open reading frames of which only the longest

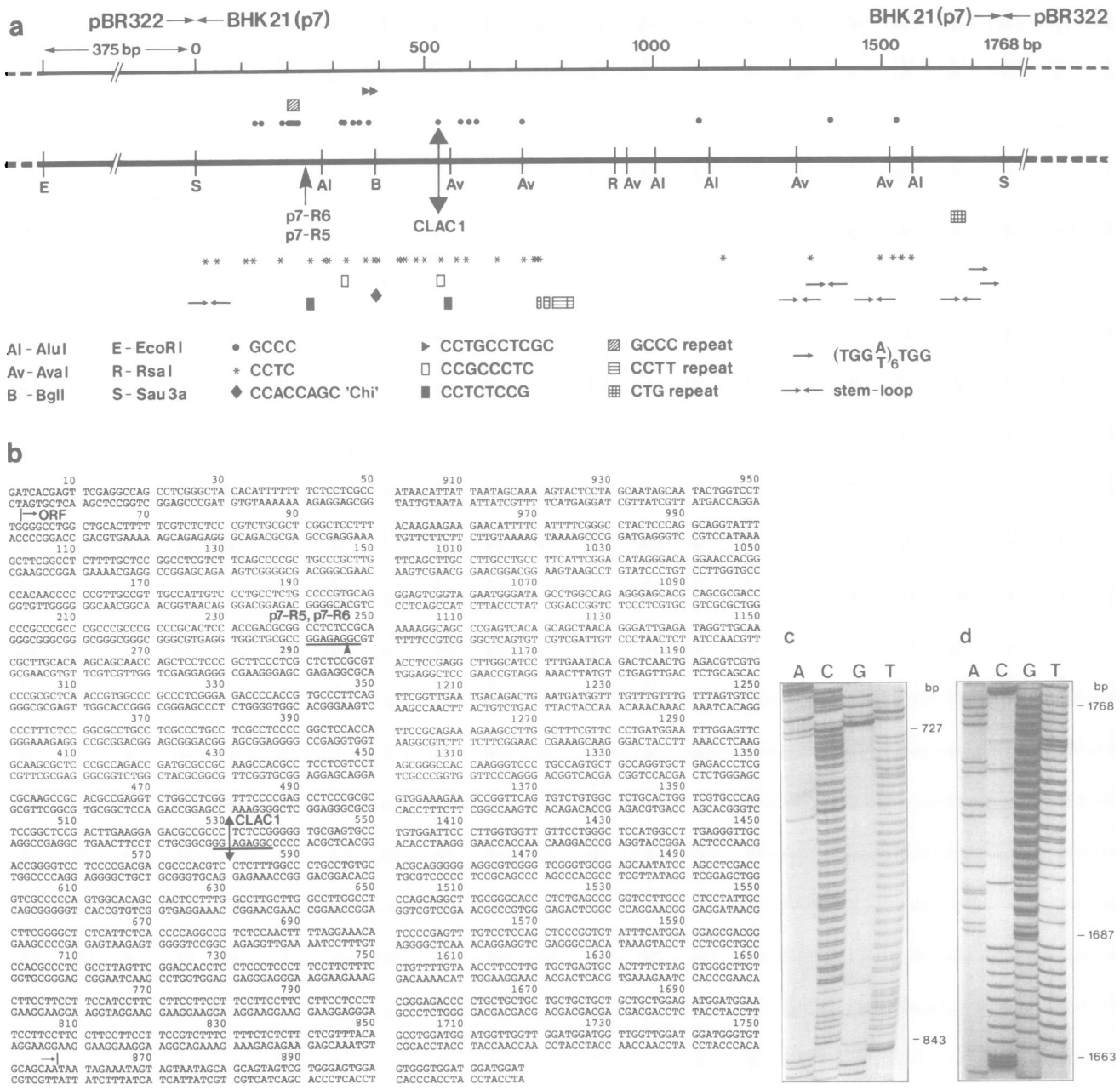


Fig. 1. Map and nucleotide sequence of the p7 hamster preinsertion DNA. (**a**) The map describes the structure of the p7 hamster preinsertion sequence as a pBR322 plasmid clone. Some of the important restriction sites are designated. The pBR322 plasmid DNA was linearized at the *Eco*RI site in some of the recombination experiments. Individual nucleotide sequences or striking repeats are indicated. The double-headed arrow marks the original site of Ad12 DNA integration as found in the Ad12-induced hamster tumour line CLAC1. The sites of recombination in the p7 DNA sequence with Ad12 DNA as observed in the *in vitro* generated recombinants p7-R5 and p7-R6 were designated by single-headed arrows. (**b**) Nucleotide sequence of the entire 1768 nt in the p7 hamster preinsertion DNA. A part of this preinsertion sequence has been published previously (Stabel and Doerfler, 1982). The first codon in the known sequence and the termination site of one of the long open reading frames (ORFs) have been marked. → ORF, first codon in the known sequence, → termination site. This ORF might extend to the left but has not been further investigated. Nucleotide numbers are arbitrary, number 1 corresponds to the G in the left *Sau*3A site (see map) of p7 DNA. (**c**) and (**d**) Autoradiograms of parts of sequencing gels. The sequence ladders present the striking CCT, CCTT, etc., repeats between nucleotide 727 and 843 (c), and the CTG repeat between nucleotides 1663 and 1687 and the TGG repeat extending to nucleotide 1768 (d).

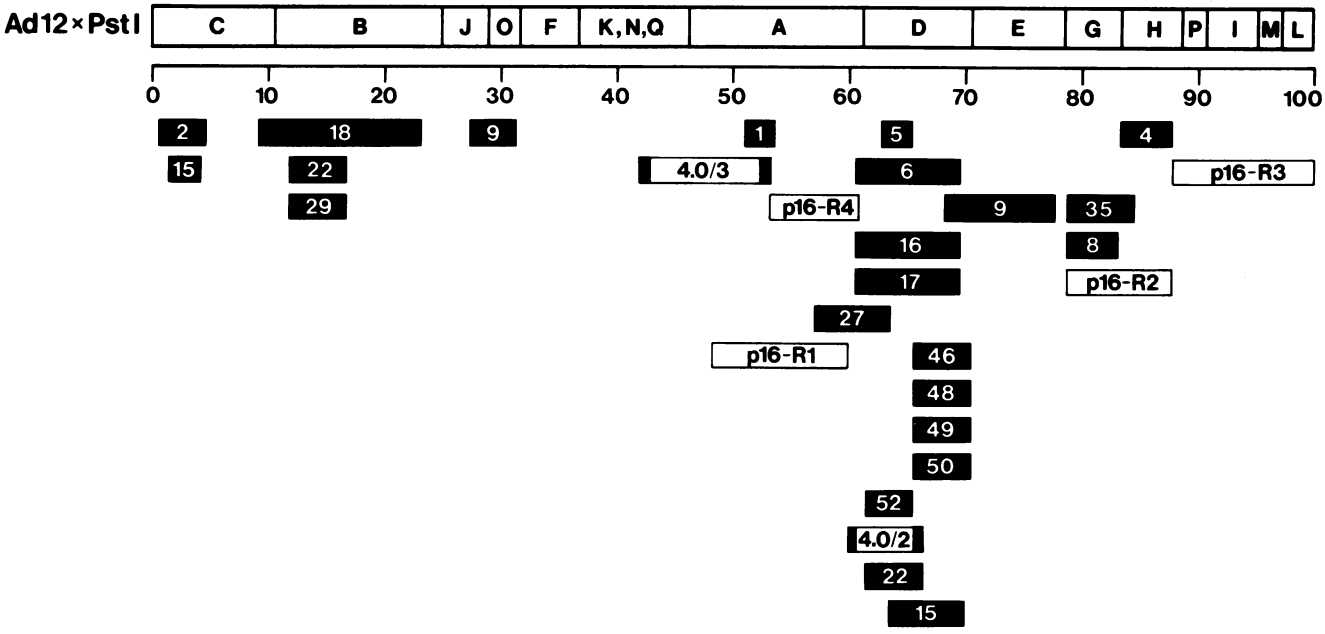
one has been reproduced here (Figure 1b). The p7 DNA sequence has no apparent homology to other Ad12 DNA junction clones previously studied in our laboratory (Doerfler *et al.*, 1983, 1985, 1987; Schellner *et al.*, 1986). The map and nucleotide sequence of the p16 preinsertion DNA has been published earlier (Lichtenberg *et al.*, 1987).

Isolation of recombinants

The generation and structure of 21 different Ad12 DNA-containing p7 recombinants, which were isolated via transfection into the recA$^-$ strain HB101/λ-LM1035 are described in Table I. In 46 different recombination experiments, 83 Ad12-positive clones (0.2%) were isolated from a total of ~38 400 ampicillin-resistant colonies. Most of the recombinants were derived from experiments in which Ad12 DNA had been cut with *Pst*I, generating 18 fragments (map at bottom of Table I), and the p7 or p16 plasmid had been linearized with the single-cutting enzyme *Eco*RI (Figure

Table I. Characteristics of some recombinants[a]

Recombinant clone	DNA substrates	Nuclear extract	Ad12 DNA component (map units)	Homology to BHK21 DNA (4.0 kbp p7) fragment	Length of insert (nucleotide pairs)
p7-R1	Ad12 x PstI + p7 x EcoRI	ultrasonic	50.5-53.5	+	2000
p7-R2			nt. 150-3950	-	3800
p7-R4		0.3 M NaCl	83-88	+	2900
p7-R5			63-65	+	2200
p7-R6			61-70	+	2000
p7-R8		ultrasonic	78-82	+	2900
p7-R9		0.3 M NaCl	71-76, 29-32	+	6100
p7-R15			8-11, 64-70	+	3000
p7-R16			61-70	-	1500
p7-R17			61-70	-	1500
p7-R18			9-25	-	ca. 3600
p7-R22			12-17, 62-67	+	2500
p7-R29			12-17	+	2300
p7-R35			78-83	+	2000
p7-R46			66-70	+	> 1000
p7-R48	Ad12 x PstI + p7/circular		66-70	+	700
p7-R49	Ad12 x PstI + p7/circular		66-70	-	n.d.
p7-R50	Ad12 x PstI + p7/DNase		66-70	+	ca. 5000
p7-R52	Ad12 x PstI x p7 x EcoRI		62-65.5	+	2200
p7-4.0/2	Ad12 x PstI + p7-4.0 x ClaI		60-67	+	ca. 2700
p7-4.0/3	Ad12 x PstI + p7-4.0 x ClaI		42-53	+	ca. 3500
p16-R1	Ad12 x PstI + p16 x SalI		48-60	+	7100
p16-R2	Ad12 x PstI + p16 x AvaI		78-88	+	1900
p16-R3	Ad12 x PstI + p16 x AvaI		88-100	+	ca. 1200
p16-R4	Ad12 x PstI + p16 x SalI		53-61	+	4200



[a]Ad12−p7 DNA recombinants recovered as pBR322 clones were designated p7-R1 to p7-R52. Ad12−p16 recombinants were named p16-R1 to p16-R4. Recombinants of Ad12 DNA with the 4.0 kbp hamster DNA segment encompassing the p7 sequence were termed p7-4.0/2 or p7-4.0/3. The entry '0.3 M NaCl' stands for the salt extraction procedure of nuclei which is described in Materials and methods. The ultrasonic extraction procedure was applied as published elsewhere (Kucherlapati *et al.*, 1985) and was used only in a few experiments. Pretreatment of the p7, p16 and Ad12 DNA substrates is outlined in the text. The schematic drawings represent the Ad12 DNA molecule and the filled-in areas designate the Ad12 DNA segments which have been included in the recombinants. A *Pst*I map of the Ad12 genome and a map unit scale are given for orientation at the bottom of the Table. The bars underneath the *Pst*I map of Ad12 DNA and the scale designate the derivation of Ad12 DNA segments in the individual recombinants (numbers inside bars). BHK-hybridization + (plus) indicates that the p7 DNA in the recombinant hybridizes to the diagnostic 4.0 kbp *Eco*RI fragment in BHK21 DNA (cf. Figure 2a). − (minus) refers to the absence of BHK21 DNA signals. The abbreviation n.d. means not determined.

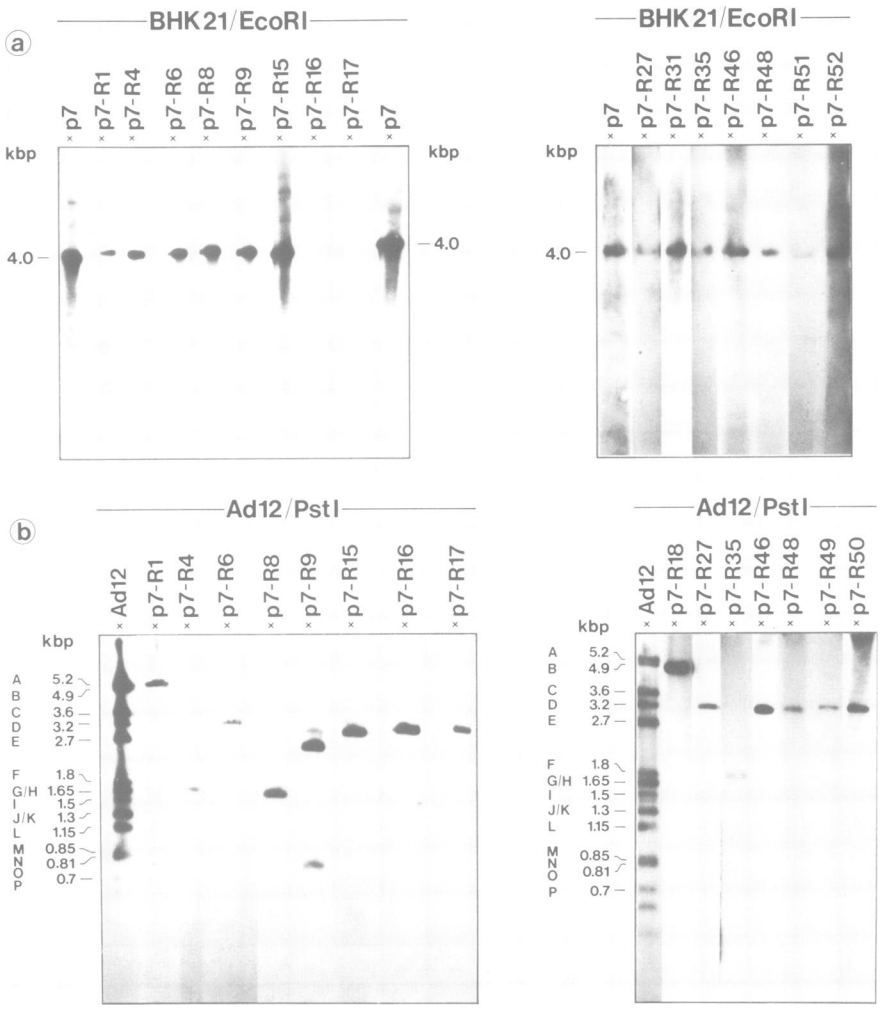


Fig. 2. Analyses of some of the Ad12−p7 DNA recombinants. (**a**) Identification of hamster DNA sequences. DNA from BHK21 hamster cells was cut with *Eco*RI, the fragments were separated by electrophoresis on a 0.6% agarose gel, and blotted to nitrocellulose filters (Southern, 1975). The DNA from individual recombinant clones as indicated was ^{32}P-labelled by nick translation (Rigby *et al.*, 1977) or by oligodeoxynucleotide labelling (Feinberg and Vogelstein, 1983), and was hybridized to different filter strips carrying BHK21 DNA fragments. In control experiments, p7 DNA was also labelled and hybridized to parallel strips in order to mark the diagnostic 4.0 kbp repetitive *Eco*RI hamster DNA fragment. (**b**) Identification of Ad12 DNA sequences. The experimental design was similar to that described in (a), except that Ad12 DNA was cut with *Pst*I, and these fragments were fixed to filter strips. Individual strips were hybridized with ^{32}P-labelled DNA from the recombinants as indicated. As a size marker, *Pst*I-cut Ad12 DNA was co-electrophoresed and hybridized to ^{32}P-labelled Ad12 DNA. Individual Ad12 DNA fragments were alphabetically designated and their sizes were indicated in kbp.

1). In the production of three recombinants, the p7 DNA was uncleaved circular, in that of three recombinants it was treated with DNase I (10 ng/ml for 2 min at 0°C). The three Ad12-positive clones isolated from experiments, in which DNase I-treated p7 DNA was used, were selected from 5000 colonies. Almost all of the recombinants were generated in salt extracts of BHK21 nuclei, and were isolated from ~38 400 colonies, three of the recombinants were produced in ultrasonic extracts (Kucherlapati *et al.*, 1985). When circular p7 DNA was used, three independent Ad12 positive clones were isolated from among 16 000 colonies. Recombinants were not generated when the cytoplasmic fraction was used. Recombinants p7-R48 and p7-R49 originated from experiments with nuclear extracts from BHK21 cells at 2 h after infection with Ad12 (50 p.f.u./cell). The lengths of the inserts of Ad12 DNA plus hamster DNA or of Ad12 DNA alone in the recombinant plasmids ranged from 700 to 7100 bp. The inserts were nearly all different in size (Table I). Recombinants of Ad12 DNA with the 4363 bp pBR322 part of the p7 construct were not observed, even though this target had 2.47 times the size of the 1768 bp hamster DNA insert. Of course, the conservation of the origin of replication and the site of ampicillin resistance in pBR322 DNA (Sutcliffe, 1978) were selected for during plasmid isolation.

Modification of reaction conditions

The experimental conditions for the recombination experiments could be varied to some extent without compromising the efficiency of the system. The time of incubation could be reduced to 10 min, KCl could be omitted from the reaction mixture, or the NaCl concentration could be lowered to 100 mM. The addition of dideoxynucleotides did not inhibit the reaction (data not shown).

As a recombination partner for Ad12 DNA a 4.0 kbp

Table II. Summary of control experiments[a] and absence of recombination between Ad12 DNA and four randomly selected BHK21 hamster DNA sequences[b]

Type of experiment	DNA substrates	Nuclear extract	Experimental details	Number of amp^R colonies tested	Ad12 DNA positive colonies
1	Ad12 × *Pst*I + p7 × *Eco*RI	0.3 M NaCl	Extract was proteinase K[c] treated	4000	0
2	Ad12 × *Pst*I + p7 × *Eco*RI	none	–	4 350	0
3	Ad12 × *Pst*I + p7 × *Eco*RI	0.3 M NaCl	Extract was made 50 mM EDTA	350	0
4	Ad12 × *Pst*I + p7 × *Eco*RI	0.3 M NaCl	DNA substrates separately[d] incubated with extract	24 000	0
5	Ad12 × *Pst*I + pBR322 × *Eco*RI	0.3 M NaCl	–	1 880	0
6	Ad12 × *Pst*I + p7 uncut	none	recA$^-$ bacteria were directly[e] transfected with DNA mixture	40 000	0
7	Ad12 × *Pst*I + p7 uncut +BHK21[f]	none	recA$^-$ bacteria were directly[e] transfected with DNA mixture	60 000	0
8	p7	none	Direct transfection of recA$^-$[e]	37 000	0
					M13 DNA positive colonies
9	M13mp9 × *Cla*I + p7 × *Eco*RI	0.3 M NaCl	–	710	0
	M13mp9 × *Pst*I + p7 × *Eco*RI	0.3 M NaCl	–	2000	0

Designation of hamster DNA clone	Size of insert	Abundance in hamster genome	Colonies tested	Ad12-positive recombinants
pBHK-1	~5.4 kbp	unique	2419	0
pBHK-2	~3.2 kbp	unique	1000	0
pBHK-3	~6.2 kbp	low repetitive	837	0
pBHK-4	~1.5 kbp	low repetitive	1500	0

[a]Experimental conditions were similar to those described in the legend in Table I.
[b]Recombination experiments were performed as described for plasmid p7 recombinants, except that plasmid clones pBHK-1 and pBHK-4 were linearized with *Cla*I. The abundance of individual hamster DNA inserts was estimated by hybridizing the ^{32}P-labelled plasmid DNA (Feinberg and Vogelstein, 1983) against *Eco*RI-generated fragments of BHK21 DNA which had been electrophoretically separated on a 0.6% agarose gel and transferred to a nitrocellulose filter (Southern, 1975).
[c]In this experiment the nuclear extract was treated with proteinase K (100 μg/ml) and 0.5% SDS for 30 min prior to being incubated with the DNA substrates. Subsequently, the DNA was re-extracted and transfected into the recA$^-$ *E.coli* strain HB101/λ-LM1035 which was used in all control experiments.
[d]In this series of experiments, the two DNA substrates were separately incubated with nuclear extract, the DNA was re-extracted and subsequently transfected into the recA$^-$ strain.
[e]In these experiments, DNA mixtures as indicated or p7 DNA by itself were directly transfected into recA$^-$ *E.coli* cells.
[f]The mixture consisted of 0.6 μg of Ad12 DNA, 0.1 μg of p7 DNA and 5 μg of BHK21 DNA.

hamster DNA fragment was also chosen that contained the 1768 bp p7 sequence in the context of flanking hamster sequences at the preinsertion site. Plasmid p7-4.0 kbp also recombined with Ad12 DNA (Table I, p7-4.0/2 and 3).

When the Mg^{2+} concentration was lowered or when the extract was made up to 12 mM EDTA, fewer Ad12-positive recombinants were observed. Omission of ATP from the reaction mixture abolished recombinants as tested with 1000 ampicillin-resistant colonies. Omission of Zn^{2+} was still compatible with recombination, but the addition of 1 mM $ZnCl_2$ to the reaction mixture yielded a >5-fold higher yield of colonies.

To exclude the possibility that Ad12-positive colonies had been generated by linearized p7 DNA being non-covalently associated with Ad12 sequences, the p7–Ad12 DNA mixture was heated to 95°C for 5 min after completion of the *in vitro* reaction and prior to transfection into the recA$^-$ *Escherichia coli* strain. Ad12-positive recombinants were still observed.

Characterization of 21 independently isolated Ad12–p7 DNA and of four Ad12–p16 DNA recombinants

The p7 hamster sequence hybridized to a 4.0 kbp *Eco*RI or *Bam*HI fragment of repetitive sequences from BHK21 DNA (Stabel and Doerfler, 1982). This cleavage pattern was exploited to assign unequivocally the p7 hamster cell origin of part of the DNA sequence in the recombinants. BHK21 DNA was cleaved with *Eco*RI, the fragments were electrophoretically separated, blotted (Southern, 1975), and each ^{32}P-labelled (Rigby *et al.*, 1977; Feinberg and Vogelstein, 1983) recombinant DNA was separately hybridized to such BHK21 DNA blots on nitrocellulose filters. All but five of the recombinants (p7-R2, p7-R16, p7-R17, p7-R18 and p7-R49) hybridized to the 4.0 kbp BHK21 DNA band (Figure 2a, Table I) and thus contained p7 DNA or parts of it. In a similar way, hybridization to BHK21 hamster DNA was also demonstrated for the four p16–Ad12 DNA recombinants (Table I).

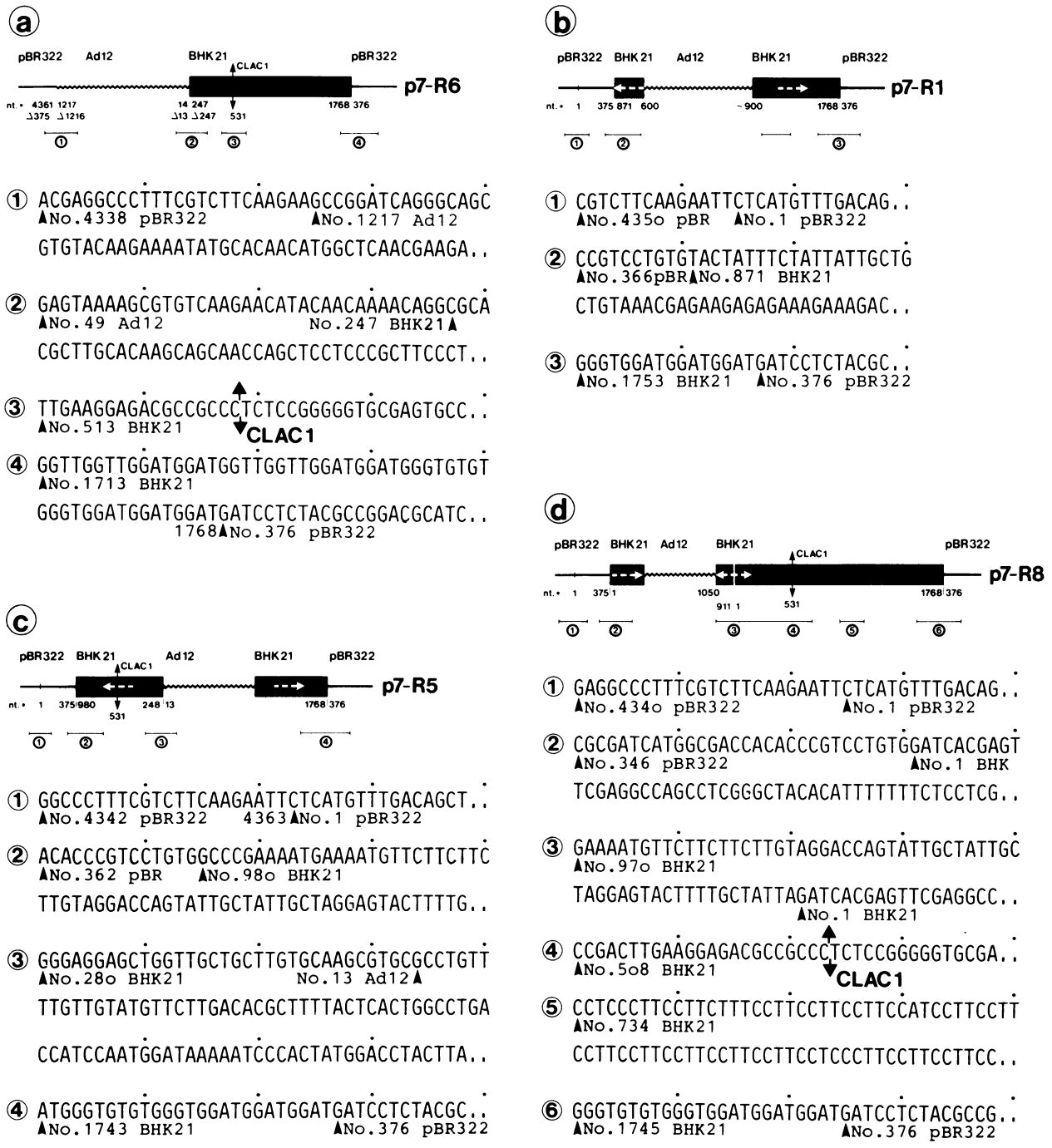


Fig. 3. Nucleotide sequences and maps of Ad12−p7 recombinants p7-R1, p7-R5, p7-R6 and p7-R8. The isolation and preliminary characterization of recombinants p7-R1 (**b**), p7-R5 (**c**), p7-R6 (**a**) and p7-R8 (**d**) was described in Table I, and Figure 2. The numbered brackets in the maps span the nucleotide sequences which were actually determined. Nucleotide (nt) numbers in the schemes related to the numbers in the pBR322 DNA sequence (Sutcliffe, 1978), to the p7 BHK21 DNA sequence (Figure 1) or to Ad12 DNA sequences (Kruijer *et al.*, 1983). Arrows inside the p7 (BHK21) DNA blocks indicated the polarity of the p7 sequence relative to the sequence shown in Figure 1. The double headed arrows (CLAC1) in the p7-R6 and p7-R8 sequences designated the site of the junction with Ad12 DNA in the DNA of the Ad12-induced hamster tumour line CLAC1 (Stabel and Doerfler, 1982). The symbol Δ indicated deletions in comparison to the original Ad12 or p7 DNA sequence. The nucleotide sequences correlated by circled numbers to sites which were designated by the same symbols in the maps. To facilitate the orientation on the Ad12 genome in (a) and (c), parts of the nucleotide sequences of the 3200 bp *Pst*I-D fragment of Ad12 DNA (cf. map in Table I) are reproduced here. The viral DNA sequences present in the recombinants are underlined:

nt 1−50

CTGCAGCGTG CGGCCTGTTT TGTTGTATGT TCTTGACACG CTTTTACTCA

nt 1181−1230

TGTGCATATT TTCTTGTACA CGCTGCCCTG ATCCGGCAAA AAACGAAAGG

The Ad12 DNA segments represented in individual recombinants were identified by restricting Ad12 DNA with *Pst*I. The *Pst*I map of Ad12 DNA had previously been determined (Kruczek and Doerfler, 1982). Each recombinant DNA was ^{32}P-labelled as described above, and hybridized to the *Pst*I fragments of Ad12 DNA which had been electrophoretically separated and transferred to nitrocellulose filters. Some of these results are shown in Figure 2b; all the data are summarized schematically in Table I. Different parts of the Ad12 genome have recombined with p7 or p16 hamster DNA in the different recombinants (Table I). This finding documented the independent origin of the recombinants. The recombination results were similar, independent of whether the p7 plasmid DNA had been linearized or had been left circular (Table I). The compilation of data in Table I implies a clustering of recombination sites in the *Pst*I-D fragment (map units 61−71) of Ad12 DNA for at least 13 independently generated recombinants. It is uncertain whether this finding is biologically significant. Comparisons of the nucleotide sequences in the p7 sequence and the *Pst*I-D fragment of Ad12 DNA with computer programs BESTFIT and FIND did not reveal sequence homologies.

Control experiments

Control experiments were performed to ascertain the independent origin of the recombinants and their generation during the incubation with nuclear extracts (Table II). Ad12 DNA-positive recombinants were not observed when a mixture of the two substrates was incubated with proteinase K-pretreated extracts (Table II, experiment 1), without extracts (experiment 2) or with extracts rendered 50 mM EDTA (experiment 3). When the two substrates were separately incubated with nuclear extracts, and when the DNA was re-extracted and transfected into recA$^-$ *E.coli*, Ad12 DNA-positive colonies were not generated (experiment 4). Moreover, when mixtures of Ad12 DNA and circular or linearized p7 DNA (experiment 6) by themselves or jointly with BHK21 cell DNA (experiment 7) were directly transfected into *E.coli*, Ad12 recombinants were not isolated. These results establish that the Ad12−p7 DNA recombinants originate during incubation with nuclear extracts. Transfection of p7 DNA into *E.coli* cells did not give rise to Ad12 DNA-positive colonies (experiment 8). Hence, there was no evidence for the presence of Ad12 DNA-containing plasmids in the p7 DNA preparation as inadvertant contaminants.

Under the *in vitro* recombination conditions tested, Ad12 DNA did not detectably recombine with pBR322 DNA (Table II, experiment 5). Nor did phage M13 DNA (Table II, experiment 9) recombine with p7 DNA.

Four randomly selected hamster DNA sequences did not recombine with Ad12 DNA in nuclear extracts

Did randomly selected hamster DNA sequences also show the capacity to recombine with Ad12 DNA in nuclear extracts at about the same frequency as the p7 hamster preinsertion sequence? The plasmids pBHK-1, pBHK-2, pBHK-3 and pBHK-4 carried hamster DNA inserts of ~5.4, 3.2, 6.2 and 1.5 kb, respectively, of unique or low repetitive origin (Table II, at bottom). These sequences were randomly selected after cloning *Bam*HI fragments of BHK21 DNA into the *Bam*HI site of pBR322 DNA and had no relation to the preinsertion sequences p7 and p16. Recombination experiments with Ad12 DNA were carried out as with p7 DNA. The randomly selected hamster DNA sequences did not yield recombinants at frequencies comparable to the p7 hamster preinsertion sequence (Table II, at bottom). The randomly isolated hamster control sequences together encompassed a 9-times larger segment of the hamster genome than the p7 preinsertion site. It was unlikely that the randomly selected hamster sequences would not recombine at all with Ad12 DNA but if they did, the frequency would not be comparable to that of recombinations with p7 or p16 DNA.

Restriction enzyme analyses of Ad12−p7 recombinant DNAs

By using a number of diagnostic restriction endonucleases, whose sites had been identified both in the p7 and the Ad12 DNA sequences, 15 recombinants were analysed and their restriction maps were determined by conventional methods (data not presented). It was apparent that the recombinants p7-R1, p7-R4, p7-R5, p7-R6, p7-R8, p7-R9, p7-R15, p7-R35 and p7-R52 carried the *Bgl*I−*Rsa*I−*Ava*II assembly of p7 hamster DNA or at least a part of it. The presence of this segment of the hamster cell p7 sequence localized the site of recombination between p7 hamster DNA and Ad12 DNA to sites different from the insertion site in cell line CLAC1 (cf. Figures 1 and 3).

Nucleotide sequence analyses on recombinants p7-R1, p7-R5, p7-R6 and p7-R8

By using synthetic oligodeoxynucleotides and the dideoxy sequencing method (Sanger *et al.*, 1977) directly on the recombinant plasmid preparations (Wallace *et al.*, 1981), nucleotide sequences were determined at sites of transition between Ad12 DNA and hamster p7 DNA or pBR322 DNA (Figure 3). As shown in Table I, p7-R1 carried Ad12 DNA sequences from the *Pst*I-A fragment, p7-R5 and p7-R6 from the *Pst*I-D fragment, and p7-R8 from the *Pst*I-G fragment. The nucleotide sequence of the *Pst*I-D fragment was published elsewhere (Kruijer *et al.*, 1983), relevant sequences from the *Pst*I-G fragment were determined in our laboratory (cf. legend to Figure 3). The nucleotide sequence-derived structures of recombinants p7-R1, p7-R5, p7-R6 and p7-R8 documented the following (Figure 3).

(i) Ad12 DNA sequences were flanked by p7 hamster DNA sequences (p7-R1, p7-R5, p7-R8) or linked to p7 hamster and pBR322 sequences (p7-R6). These sequence arrangements precluded the possibility that the DNA molecules, which were added to the reaction mixture, were simply end-to-end joined, but rather were true recombinants.

(ii) The sites of linkage between Ad12 DNA and hamster DNA were distinct from the site of linkage in the CLAC1 junction clone (Figures 1 and 3).

(iii) In some of the recombinants (p7-R1, p7-R5, p7-R8), complex hamster DNA rearrangements were observed which were in part reminiscent of cellular and viral DNA fragment transpositions which were recently described in an Ad12-induced hamster tumour (Lichtenberg *et al.*, 1987) and

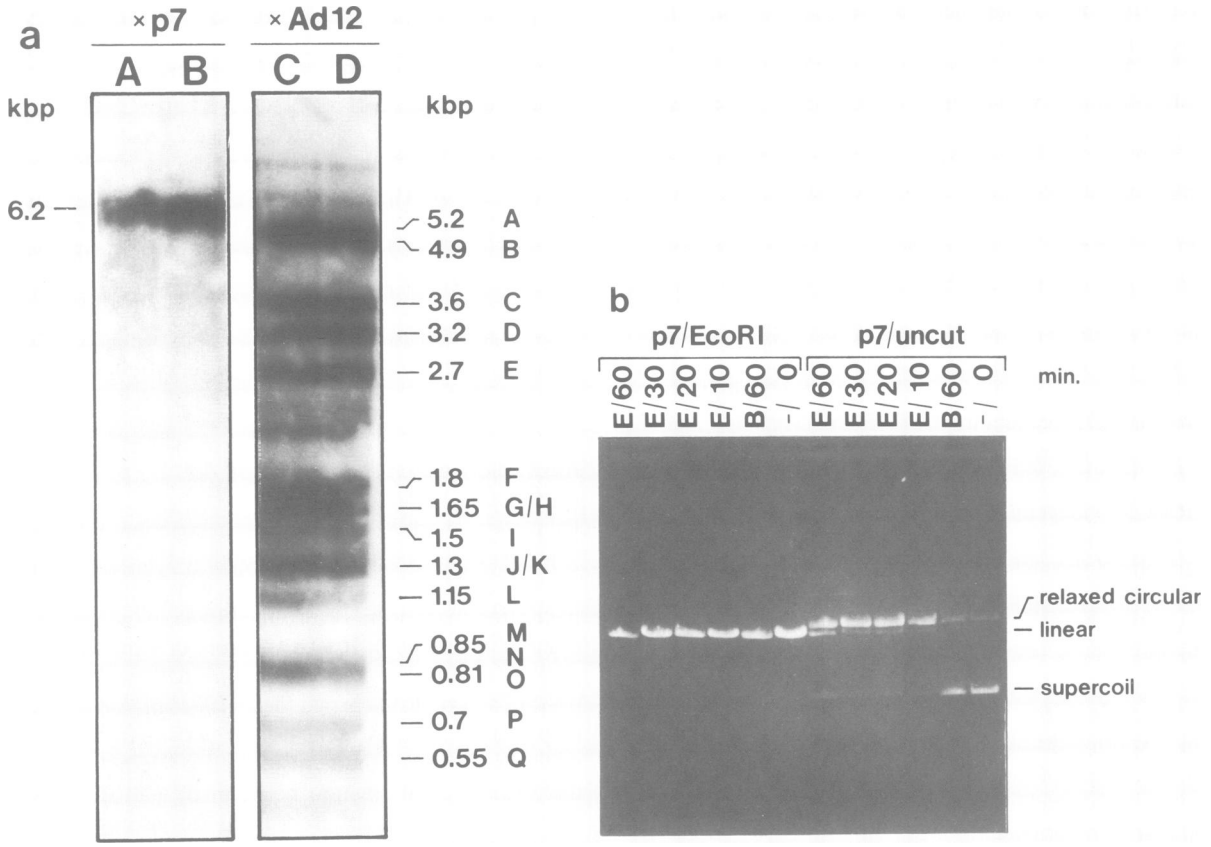


Fig. 4. Stability of the *Eco*RI-cut p7 DNA and the *Pst*I-cut Ad12 DNA fragments in nuclear extracts of BHK21 cells. (**a**) Experimental details are described in the text. The blotted DNA fragments were derived from experiments in which they had been incubated with extracts (A and C) or from controls (B and D) and were hybridized to ^{32}P-labelled p7 DNA (A and B) or to Ad12 DNA (C and D). An autoradiogram is shown. (**b**) UV-light photograph of an ethidium bromide stained gel. p7 DNA was incubated in the reaction buffer devoid of nuclear extracts (B) or in nuclear extracts (E) for periods as indicated. Uncut or *Eco*RI circularized p7 DNA was used as indicated. After the incubation period, the DNA was re-extracted. Untreated circular and linearized DNAs (−/0 min) were co-electrophoresed on a 0.6% agarose gel. The positions of supercoiled circular, relaxed circular and linear DNAs were indicated.

in the Ad12-transformed cell line HA12/7 (Jessberger *et al.*, 1989).

(iv) It was striking that recombination between Ad12 DNA and the p7 plasmid DNA, in general involved hamster DNA and not plasmid DNA sequences, although in p7-R6 one end of Ad12 DNA was linked to pBR322 DNA.

The structures of the described recombinants revealed that complex recombination reactions had occurred between Ad12 DNA and p7 DNA in a cell-free system. These reactions might encompass elements of viral DNA integration.

Are the input DNAs stable in the cell-free recombination system?

In a volume of 100 μl of nuclear extract, 0.5 μg of *Pst*I-cut Ad12 DNA and 0.5 μg of *Eco*RI-cut p7 DNA were incubated for 60 min (Figure 4a, A and C). Subsequently, the DNA was extracted, electrophoresed on a 1.0% agarose gel, denatured, blotted to a nitrocellulose filter and hybridized to ^{32}P-labelled p7 DNA followed by autoradiography (Figure 4a, A and B). The labelled DNA was then removed by washing (100°C, 0.1 × SSC, 0.1% SDS), and the DNA on the filters was hybridized to ^{32}P-labelled Ad12 DNA Figure 4a, C and D). As a control, a mixture of *Pst*I-cut Ad12 DNA and *Eco*RI-cut p7 DNA was treated similarly without being incubated with nuclear extracts (Figure 4a, B and D). The autoradiograms in Figure 4a demonstrated that incubation with nuclear extracts did not extensively degrade the DNA preparations. Similarly, when uncleaved or *Eco*RI-linearized p7 DNA was incubated with extract (E) or in the reaction buffer without nuclear extracts (B) for periods between 10 and 60 min, evidence for DNA degradation was not apparent (Figure 4b), except that the bulk of the supercoiled form of p7 DNA was converted to the relaxed circular form and partly to the linear form of p7 DNA.

Discussion

Modalities of the cell-free recombination system

A system was developed which used nuclear extracts from hamster BHK21 cells. This system catalysed recombination between Ad12 DNA and the p7 or the p16 hamster preinsertion sequence in an *in vitro* reaction. The experiments described here were designed to demonstrate that the p7 and the p16 preinsertion sequences were capable of recombining in a cell-free extract with non-homologous Ad12 DNA. Elements in the p7 or p16 hamster sequence might be recognized by cellular proteins. The recombination reaction could not be elicited when uncut Ad12 DNA was used, perhaps because it was too long to be effectively inserted into and maintained in the p7 or p16 plasmid. Recombination was observed both with uncut circular and *Eco*RI-linearized p7 DNA. The importance of DNA termini

in some recombination systems was demonstrated earlier (Wilson *et al.*, 1982; Roth *et al.*, 1985; Roth and Wilson, 1986). Nuclear extracts similar to those utilized in the present experiments were previously used by other investigators (Darby and Blattner, 1984; Kucherlapati *et al.*, 1985) who successfully rejoined DNA molecules carrying homologous sequences. In other work, cytoplasmic extracts were employed (Brown *et al.*, 1987).

Ad12 DNA sequences were inserted into p7 hamster DNA as evidenced by the nucleotide sequence data obtained for recombinants p7-R1, p7-R5 and p7-R8 (Figure 3).

Striking sequence repeats were observed in the p7 preinsertion sequence (Figure 1). Although their role in serving as landmarks for the recombination reaction remained uncertain, it should be mentioned that the CCTT repeat was also observed, e.g. in the mouse $\varkappa$-immunoglobulin gene (Hoechtl and Zachau, 1983), in the mouse MHC class II H2-Ia-β gene (Larhammar *et al.*, 1983), in the hamster alpha A crystalline gene (Van den Heuvel *et al.*, 1985), and in the human HLA-DP-β1 and α1 genes (Kelly and Trowsdale, 1985). Twelve of the *in vitro* produced recombinants carried Ad12 DNA sequences from the *Pst*I-D fragment of the viral genome, i.e. map units 61–71 (Table I). We do not yet understand the significance of these findings.

Control experiments confirmed that recombination had actually taken place during incubation with nuclear extracts (Table II). The negative outcome of these experiments and the fact that the sites of recombination between Ad12 DNA and the p7 or p16 hamster DNA were different among each other and from the CLAC1 or T1111(2) viral–cellular junction site, respectively, precluded that we had accidentally reisolated known junction plasmids as contaminants or that the recombinants had been generated in the intermediary *E.coli* host. The finding that the separate incubation of p7 and Ad12 DNAs and the subsequent transfection of these molecules into the recA$^-$ *E.coli* strain did not give rise to recombinants also ruled out the possibility that only part of the recombination reaction had occurred in the *E.coli* host. This interpretation was underscored by the lack of recombination between Ad12 DNA and randomly selected hamster DNA sequences.

Selectivity of the recombination reaction and resemblance to viral DNA integration

Elements of selectivity. Our evidence supports the notion that the recombination reaction has elements of selectivity. (i) The structure of the recombinants demonstrates that Ad12 DNA has recombined with the p7 hamster DNA and not with the adjacent, 2.4-fold longer pBR322 sequence. (ii) Ad12 DNA recombination has also been demonstrated with a second preinsertion site, p16. (iii) Recombinants between p7 DNA and phage M13 DNA have not been observed (Table II, experiment 9). (iv) The results compiled in Table II document that Ad12 DNA does not recombine with four different, randomly selected hamster DNA sequences of unique or repetitive type at frequencies in the range found for p7 or p16 DNA and Ad12 DNA. The randomly selected hamster sequences jointly have a size ~9-fold that of the p7 hamster sequence. It would be premature to argue that recombination between Ad12 DNA and p7 or p16 DNA was specific. Non-homologous recombination can probably occur with many different cellular sites and we have not screened millions of potential recombinant clones. However, it can be reasoned that Ad12 DNA recombines with the p7 and p16 hamster preinsertion sequence with a certain preference that has not been shown vis-à-vis randomly selected hamster DNA sequences.

Resemblance to viral DNA integration. The provisional terms 'homologous' and 'non-homologous' recombination may in the future be superseded by more realistic definitions. But for the present, the type of recombination investigated here must be considered non-homologous recombination. It is a matter of conjecture whether the integration of Ad12 DNA into the hamster chromosome is a special form of non-homologous recombination and/or is associated with more complex elements. We argue that the recombination reaction described here constitutes at least an important element of integrative recombination. The products of the *in vitro* recombination reaction bear resemblance to the outcome of several of the adenovirus integration events studied previously (Doerfler *et al.*, 1983, 1985, 1987).

Materials and methods

Cells and virus

BHK21 hamster cells were grown as monolayer cultures (75 cm^2 Falcon flasks) in Dulbecco's medium supplemented with 10% fetal bovine serum. Ad12 was propagated in suspension cultures of KB cells. Virus and viral DNA were purified as described (Doerfler *et al.*, 1972).

Plasmid DNA preparations

The pBR322 plasmid DNA contained hamster DNA preinsertion sequences as a 1768 bp insert in p7 or a 3.1 kbp insert in p16. The p7 or p16 plasmid was propagated in *E.coli* strain HB101/λ and was purified by standard methods (Clewell and Helinski, 1972).

Randomly selected BHK21 hamster DNA fragments were cloned by cleaving BHK21 hamster DNA with *Bam*HI and ligating these fragments into the *Bam*HI site of plasmid pBR322 DNA: pBHK-1 DNA (unique hamster DNA ~5.4 kbp), pBHK-2 (unique hamster DNA, ~3.2 kbp), pBHK-3 (low repetitive hamster DNA, ~6.2 kbp) and pBHK-4 (low repetitive DNA, ~1.5 kbp). In other experiments, a 4.0 kbp hamster DNA fragment was cloned into the *Eco*RI site of pBR322 DNA (Stabel and Doerfler, 1982). This 4.0 kbp fragment contained the 1768 bp p7 fragment and flanking hamster DNA sequences (clone p7-4.0).

Restriction mapping, subcloning and nucleotide sequence determination of the p7 hamster preinsertion sequence

Restriction maps of the 1768 bp hamster DNA insert in plasmid p7 were determined (cf. Figure 1). The hamster DNA was excised with *Sau*3A from the pBR322 vector and was subsequently cut with *Alu*I. The three *Alu*I and the two flanking *Alu*I–*Sau*3A hamster DNA fragments were fitted with *Bam*HI linkers and subcloned into the *Bam*HI site of the polycloning sequence in M13mp18 DNA (Norrander *et al.*, 1983). By using the commercial M13 17mer primer (BRL) or synthetic oligodeoxynucleotide primers, which were produced in a 381A DNA Synthesizer (Applied Biosystems), the p7 hamster DNA sequence or relevant parts of the p7-R1, p7-R5, p7-R6 and p7-R8 recombinant sequences were determined by the dideoxy chain termination method (Sanger *et al.*, 1977). In some experiments, sequences were obtained directly from the double-stranded plasmid preparation (Wallace *et al.*, 1981) using suitable synthetic primers.

The restriction map and nucleotide sequence of the p16 hamster DNA segment were published elsewhere (Lichtenberg *et al.*, 1987). For nucleotide sequence comparisons, published Ad12 DNA sequences were used (Sugisaki *et al.*, 1980; Bos *et al.*, 1981; Kimura *et al.*, 1981; Kruijer *et al.*, 1983). Some of the not previously published Ad12 DNA sequences were determined by using synthetic oligodeoxynucleotide primers. Programs of the University of Wisconsin Genetics Computer Group were employed in a VAX 11/750 computer (Digital Equipment Corporation).

Preparation of nuclear extracts from BHK21 cells

In the initial experiments, two different methods were tested (Darby and Blattner, 1984; Kucherlapati *et al.*, 1985). For most of the experiments described here we adapted the following procedure. About 2–3 × 10^8 cells

were washed twice in serum-free medium, scraped off the plastic support at 4°C, centrifuged and washed twice with PBS-d (PBS deficient in Mg^{2+} and Ca^{2+}). The cells were then incubated for 5–10 min in 2 ml of ice cold 10 mM Tris–HCl, pH 7.4, 10 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonylfluoride (PMSF) and 12 μg/ml of aprotinin. Subsequently, the cytoplasmic membranes were broken by 10–15 strokes in a Dounce homogenizer (S-fitting). Disruption was monitored by phase contrast microscopy. The homogenates were centrifuged at 2000 *g* for 5 min, and the nuclear pellet was resuspended in 1.0 ml of the same buffer which was made 300 mM NaCl. The nuclear suspension was kept on ice for 1 h, and subsequently centrifuged at 6°C and 16 000 g for 20 min in an Eppendorf centrifuge. The supernate was made 10% glycerol, 10 mM β-mercaptoethanol and 0.6 mM EDTA, and 100 μl aliquots were stored at −80°C.

In vitro recombination experiments

Reactions were carried out in a total volume of 100 μl. Protein concentrations were determined according to the method of Bradford (Bradford, 1976). The total volume of 100 μl was (final concentrations) 40 mM Tris–HCl, pH 7.4, 25 mM $MgCl_2$, 1 mM $ZnCl_2$, 5 mM KCl, 0.5 mM PMSF, 0.06 μg of aprotinin, 0.15 M NaCl, 5% glycerol, 5 mM β-mercaptoethanol, 0.5 mM DTT, 0.3 mM EDTA, 1 mM ATP, 5 mM creatine phosphate, 0.1 mM each of the four dNTPs, 50 μl of extract (~1 μg protein per μl), 1 μg of Ad12 DNA and 0.1 to 0.2 μg of p7 or p16 DNA pretreated as described in Table I. The reaction proceeded for 30 min at 37°C. Subsequently, the total DNA was re-extracted by the SDS–proteinase K–phenol:chloroform (1:1) method (Sutter *et al.*, 1978), ethanol precipitated and resuspended in 10 mM Tris–HCl, pH 7,5, 1 mM EDTA (TE). The OD_{280}/OD_{260} ratios of the nuclear extracts were 1.8 indicating the presence of minimal amounts of nucleic acid in the extracts.

Isolation of p7–Ad12 or p16–Ad12 DNA recombinants

DNA was transfected into the recA$^-$ *E.coli* strain HB101/λ-LM1035 by standard methods (Morrison, 1979). In a few experiments, the DNA was heated to 95°C for 5 min after reisolation from the extracts and before transfection into HB101/λ-LM1035 in order to eliminate non-covalently linked hybrid molecules. The recA$^-$ phenotype of this strain was regularly ascertained by assessing its high sensitivity towards irradiation with ultraviolet light. Ad12 DNA-positive colonies were identified by the Grunstein and Hogness (1975) technique using ^{32}P-labelled Ad12 DNA as hybridization probe. Positive colonies were purified by several single-colony isolations and plasmid DNA was purified as described (Clewell and Helinski, 1972). Recombinant DNAs were mapped by standard restriction analyses, Southern (1975) blot hybridization or nucleotide sequence analyses (Sanger *et al.*, 1977).

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Note added in proof

An additional five randomly selected hamster DNA sequences, which were not related to the p7 or p16 preinsertion sequences, were also tested for recombination with Ad12 DNA in the cell-free system. Recombinants were not observed. These five hamster DNA sequences together comprised 21 kbp and exceeded in size the p7 preinsertion sequence by a factor of 11.8. These results further supported the notion of selective recombination of Ad12 DNA *in vitro* with the preinsertion site DNAs.