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Fractionated nuclear extracts from hamster cells catalyze cell-free recombination at selective sequences between adenovirus DNA and a hamster preinsertion site

(nonhomologous insertional recombination/*in vitro* system/polymerase chain reaction/ion-exchange chromatography)

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ABSTRACT **We have explored the mechanism of adenovirus type 12 (Ad12) DNA integration because of its importance for viral oncogenesis and as an example of insertional recombination. We have used a fractionated cell-free system from nuclear extracts of hamster cells and have partly purified nuclear proteins that could catalyze *in vitro* recombination. As recombination partners, the 20,880- to 24,049-nucleotide *Pst* I D fragment of Ad12 DNA and the hamster preinsertion sequence p7 from the Ad12-induced tumor CLAC1 have proven to recombine at higher frequencies than randomly selected adenoviral or cellular DNA sequences. A preinsertion sequence might carry elements essential in eliciting recombination. Patch homologies between the recombination partners seem to play a role in the selection of sites for recombination *in vivo* and in the cell-free system. Nuclear extracts from BHK21 cells were prepared by incubating the nuclei in 0.42 M $(NH_4)_2SO_4$ and fractionated by Sephacryl S-300 gel filtration, followed by chromatography on Mono S and Mono Q columns. The purified products active in recombination contained a limited number of different protein bands, as determined by polyacrylamide gel electrophoresis and silver staining. The most highly purified fraction IV had helicase and topoisomerase I activities. We used two different methods to assess the *in vitro* generation of hamster DNA–Ad12 DNA recombinants upon incubation with the purified protein fractions: (*i*) transfection of the recombination products into recA$^-$ strains of *Escherichia coli* and (*ii*) the polymerase chain reaction by using amplification primers unique for each of the two recombination partners. In p7 hamster DNA, the nucleotide sequence 5'-CCTCTCCG-3' or similar sequences served repeatedly as a preferred recombination target for Ad12 DNA in the tumor CLAC1 and in five independent cell-free recombination experiments.**

The induction of tumors by adenovirus type 12 (Ad12) in hamsters (1) is associated with the integration of Ad12 DNA into the hamster cell genome (2, 3). The structures of several integration sites suggest that in established cell lines of Ad12-induced hamster tumor cells or of adenovirus-transformed cells a specific nucleotide sequence does not exist at which adenovirus (foreign) DNA can insert into the mammalian genome (3). Foreign DNA integrates frequently at transcriptionally active sequences in the mammalian host genome (4, 5). Under certain conditions Ad12 DNA may integrate at selective sites.

Cell-free systems have been developed to investigate genetic recombination in viral systems (6–8). We have chosen the hamster preinsertion sequence p7 (9) derived from the Ad12-induced hamster tumor CLAC1 (10) and the 20,880- to 24,049-nucleotide (21) fragment of Ad12 DNA as recombination targets (6, 8) and have purified cell-free extracts from BHK21 cell nuclei to a few protein bands retaining activity in cell-free recombination.

MATERIALS AND METHODS

Preparation and Fractionation of Nuclear Extracts. Crude nuclear extracts (fraction I) were prepared from BHK21 cells grown in suspension cultures (8). Three milliliters of fraction I (Fig. 1) was applied to a Sephacryl S-300 column (Pharmacia, XK 16/70, 50 cm long) equilibrated and developed at 30 ml/hr with buffer A [20 mM Hepes, pH 7.9/20% glycerol/150 mM NaCl/0.2 mM (each) EDTA and EGTA/2 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride]. The active fractions from the shoulder in the OD_{280} absorbancy profile (filled area in Fig. 1*a*) were pooled. This fraction II was loaded onto a Mono S column (Pharmacia HR 5/5) equilibrated with buffer A. Fractions catalyzing cell-free recombination eluted at 30 ml/hr in the flow-through of the OD_{280} profile as fraction III, which was adsorbed at 30 ml/hr onto a Mono Q column (Pharmacia HR 5/5). Recombinationally active proteins were eluted with buffer A containing 500 mM NaCl, pooled, and dialyzed against buffer A (fraction IV). Fraction IV could be frozen in liquid nitrogen and stored at −80°C. Fraction III' was obtained by passing proteins from fraction II directly over a Mono Q column at 1 M NaCl.

Transfection Assay for Recombinants. For the generation of recombinants rec50, rec55, rec32, and rec36, 0.2 μg of the *Eco*RI-linearized p7 DNA and 1 μg of the *Pst* I D fragment of Ad12 DNA [nucleotides 20,880–24,049 (21)] were incubated at 37°C for 30 min in 100 μl of 20 mM Hepes, pH 7.9/15 mM $MgCl_2$/1 mM $ZnCl_2$/75 mM NaCl/0.5 mM phenylmethylsulfonyl fluoride/10% glycerol/5 mM creatine phosphate/1 mM ATP/0.1 mM (each) of the four deoxyribonucleoside triphosphates (dNTPs) and 50 μl (0.1–0.25 μg/μl) of fraction III' or IV. Subsequently, the DNA was reextracted by the SDS/proteinase K/phenol/chloroform method. The DNA was then transfected into the recA$^-$ strain HB101/LM1035 of *Escherichia coli* and analyzed as described (6, 8, 11).

Analysis of Recombinants by the Polymerase Chain Reaction (PCR). The PCR (12) mixture used for amplification contained 10 pg of template DNA, 0.5 μM primers, 0.2 mM of the four dNTPs, 3 mM $MgCl_2$, 50 mM KCl, 10 mM Tris·HCl (pH 9.0), 0.1% gelatin, 0.1% Triton X-100 (wt/vol), and 2.5 units of Taq DNA polymerase (Promega). The cycle conditions were 1 min at 94°C for denaturation, 2 min at 68°C for annealing, 2 min at 72°C for polymerization for 10 cycles; this was followed by 25 cycles at an annealing temperature of 60°C. Amplification products were analyzed by electrophoresis on a 0.8% agarose gel in TBE (0.1 M Tris·HCl/77 mM

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Abbreviations: Ad12, adenovirus type 12; oligo, oligodeoxyribonucleotide.

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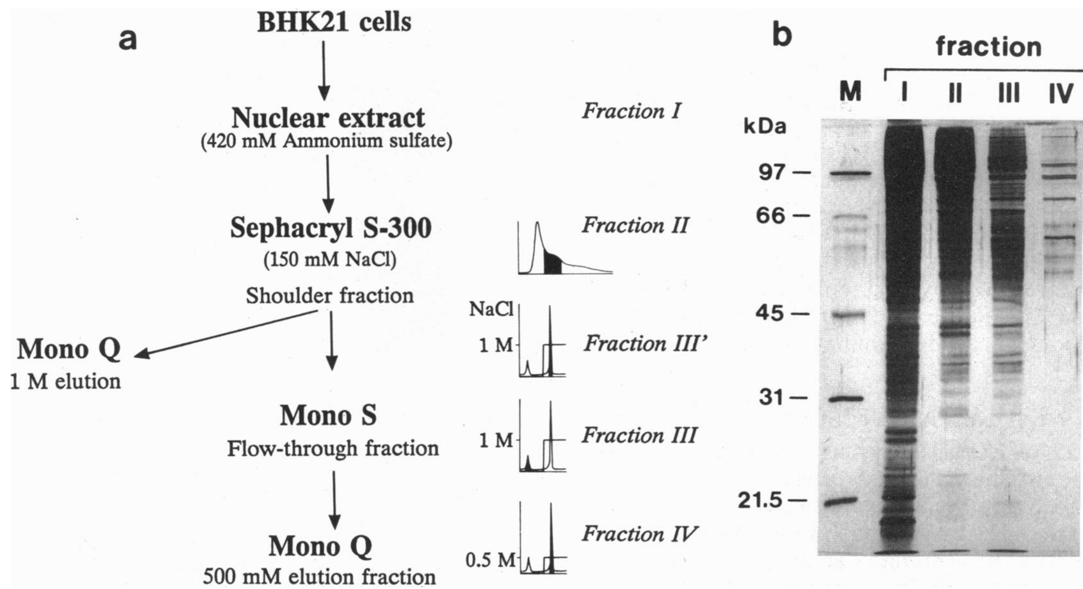

FIG. 1. (*a*) Fractionation of nuclear extracts from BHK21 hamster cells. Fractionation of crude nuclear extracts by gel filtration on Sephacryl S-300 followed by chromatography on Mono S and Mono Q columns is described in the text. Elution profiles of each column are presented schematically, and the active fractions are indicated by filled areas. The NaCl concentration is also shown. (*b*) Protein analyses in silver-stained SDS/polyacrylamide gels of fractions I–IV of nuclear extracts from BHK21 hamster cells. The proteins in these fractions were analyzed by electrophoresis in polyacrylamide gels followed by silver staining (14). In lane M, marker proteins (Bio-Rad, silver stain markers) of known molecular masses (kDa) were coelectrophoresed.

borate/2.5 mM EDTA). *Ava* I- or *Rsa* I-cut p7 DNA was used as size marker (in bp).

Electrophoretic Mobility Shift Assay. This method has been described (8).

Analysis of Proteins by Electrophoresis on SDS/Polyacrylamide Gels. Proteins resolved by electrophoresis (13) on 0.1% SDS/8% polyacrylamide gels (polyacrylamide/bisacrylamide, 19:1) were subsequently silver stained (14).

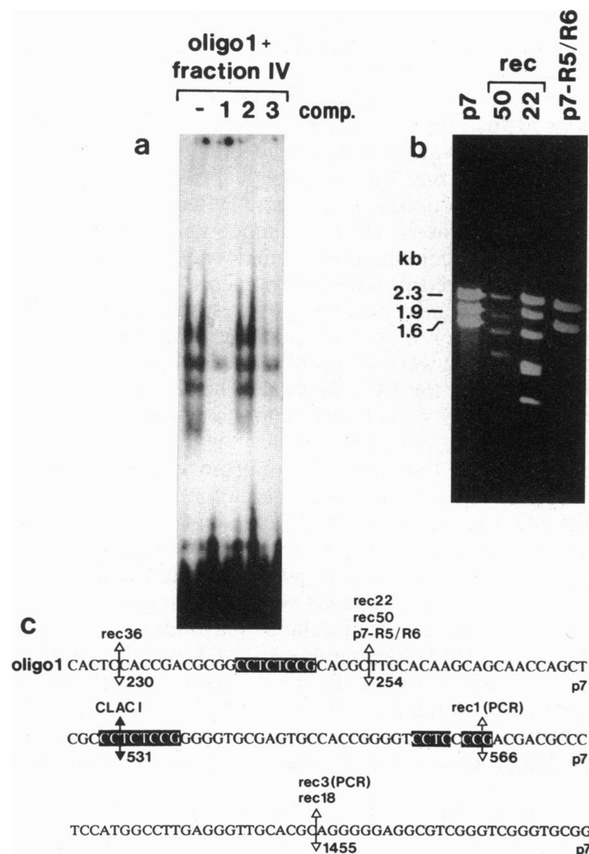

FIG. 2. Characteristics of recombinants generated in cell-free recombination experiments. (*a*) Electrophoretic mobility shift assay of proteins from fraction IV with oligo 1 [lane —, without competitor (comp.)], which represents the sequence shown in *c* (top). This delayed migration can be inhibited by the same unlabeled oligo (lane comp. 1), attesting to the specificity of this DNA–protein interaction. Lane comp. 2, the synthetic oligo 5′-AGTCCGCGGCTCCGGGGT-TGAAACTCACTCCGGGGCTGTGGACGTCGGCT-3′, comprising the nucleotide sequence from −37 to +13 in the late E2A promoter of Ad2 DNA (16), was used as competitor. Lane comp. 3, the p7 sequence between nucleotides 511 and 550 at the CLAC1 junction (6) in p7 DNA, 5′-ACTTGAAGGAGACGCCGCCCTCTCCGGGGGT-GCGAGTGCC-3′, was used as competitor. In all experiments, 50 ng of competitor was used. (*b*) UV photograph of a gel on which the *Bgl* I cleavage patterns of p7 DNA, rec50, rec22, and p7-R5/R6 DNAs are compared. The patterns of rec50, rec22, and p7-R5/R6 DNAs differ distinctly. (*c*) Nucleotide sequences at the sites of linkage of Ad12 DNA to p7 DNA were determined for recombinants rec36 and rec50 by published methods (17). The sequence in rec22 was published earlier (8). Recombination between p7 and Ad12 DNAs in rec22, rec50, and p7-R5/R6 (6) was at the same site in p7 DNA, although the recombinants differed otherwise significantly in their structures (see *b*). Another recombinant (rec36) was generated by incubation with a Mono Q fraction (fraction III′) that was not purified over a Mono S column. The rec22, rec50, and p7-R5/R6 site in p7 DNA had previously served as target sequence in cell-free recombinations with Ad12 DNA, when crude nuclear extracts (fraction I) were incubated with the recombination partners to yield recombinants p7-R5/R6 (6). The sites indicated by open double-headed arrows functioned as recombination targets in cell-free recombination experiments. The numbers below the p7 nucleotide sequence refer to the published nucleotide sequence (6). Both sites of recombination shown were located in the vicinity of the sequence 5′-CCTCTCCG-3′ (black background). The nucleotide sequences determined at the sites of recombination in the PCR-identified recombinants rec1(PCR) and rec3(PCR) were also reproduced (*c*). The PCR-amplified DNA fragments which, hybridized to both reaction partners, were gel purified, excised from the 0.8% agarose gel, eluted, and ethanol precipitated. Subsequently, the nucleotide sequence of part of the DNA fragment was determined (17). The rec1(PCR) site lies in close proximity to the original site of Ad12 DNA integration in the Ad12-induced tumor CLAC1 (closed arrowheads).

Assays for Enzymatic Activities. Recombination-active fraction IV was tested for helicase and topoisomerase I. Assay conditions for helicase have been described (8, 15). For topoisomerase I assays, 600 ng of p7 DNA was incubated for 8 min at 37°C in a volume of 20 μl of 50 mM Tris·HCl, pH 7.5/50 mM KCl/10 mM $MgCl_2$/0.1 mM EDTA/0.5 mM dithiothreitol/30 μg of bovine serum albumin per ml containing different amounts of proteins from fraction IV (Fig. 3*a*). The reaction was terminated in 0.1% SDS/10 mM EDTA/4% glycerol by heating for 10 min at 65°C. Subsequently, reaction products were resolved by electrophoresis in 0.6% agarose gels in 0.04 M Tris-acetate, pH 8.0/1 mM EDTA and visualized by ethidium bromide staining followed by UV photography.

RESULTS AND DISCUSSION

Fractionation of Extracts and Characterization of Purified Fractions. The enzymatic activities promoting cell-free recombination between the p7 segment of hamster DNA and the *Pst* I D fragment of Ad12 DNA eluted in the shoulder fractions (fraction II) of the OD_{280} profile from the Sephacryl S-300 gel filtration column (Fig. 1*a*). In subsequent purification steps, the activity resided in the flow-through of the FPLC Mono S column (fraction III) and could be eluted with 0.5 M NaCl from the FPLC Mono Q column (fraction IV). The protein composition of fractions I–IV was determined by electrophoresis on SDS/polyacrylamide gels, and protein bands were visualized by silver staining (14). Fraction IV contains only a limited number of polypeptide bands (Fig. 1*b*). By step or gradient elution using up to 0.4 M NaCl on the Mono Q column, fraction IV could not, so far, be purified further. The proteins involved in cell-free recombination thus appear to be tightly bound in a complex resistant to 0.4 M NaCl.

Proteins in the recombinationally active fraction IV bound specifically to a double-stranded 50-nucleotide oligodeoxyribonucleotide (oligo) (Fig. 2*a*, lane —) that contained the sequence 5′-CCTCTCCG-3′ and was derived from the p7 sequence (Fig. 2*c*, oligo 1). It carried multiple sites of cell-free recombination. Binding could be specifically inhibited by the same oligo (Fig. 2*a*, lane 1) or by the 40-nucleotide synthetic sequence spanning the site of integration in cell line CLAC1 (Fig. 2*a*, lane 3). There was no competition when nonspecific poly(dA-dT):poly(dA-dT) (data not shown) or the −37 to +13 oligo from the unrelated sequence of the E2A late promoter of Ad2 DNA (16) was added as competitor (Fig. 2*a*, lane 2). The sequence in oligo 1 served as the acceptor target for Ad12 DNA recombination in the generation of seven recombinants (Fig. 2*c*), of which six were produced by cell-free recombination experiments, one in the transformation event leading to the Ad12-induced tumor cell line CLAC1. The same oligo 1 had high specific binding activity for proteins in the recombinationally active fractions I–IV (see Fig. 1*a*).

Known Enzymatic Activities in Fraction IV. Fraction IV contained topoisomerase I (Fig. 3*a*) and helicase activities (Fig. 3*b*). Increasing amounts of proteins from fraction IV elicited increased levels of enzymatic activities. Both enzymatic functions are thought to be involved in recombination. Helicase activity has previously been found in fraction II (8).

Identification of Cell-Free Recombinants. Recombinants generated by fractions II–IV were identified by two different methods. Transfection of recombination products into the recA⁻ strain HB101/LM1035 of *E. coli* and identification of Ad12 DNA-positive colonies by hybridization to a ^{32}P-labeled probe (11) were used earlier (6, 8). In some of these recombinants, the nucleotide sequence across the junction between Ad12 DNA and the p7 hamster DNA sequence was determined (Fig. 2*c*; see Fig. 5). Table 1 summarizes all recombinants isolated with this assay and presents a quantitative evaluation of the data. For unknown reasons, fractions III′ and IV yielded more recombinants than the less highly purified fractions.

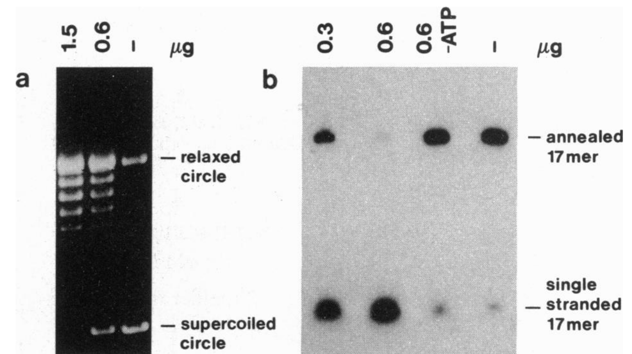

FIG. 3. Topoisomerase I (*a*) and helicase (*b*) activities in fraction IV. The amount of protein (μg) used in each assay is indicated. The helicase reaction (*b*) is dependent on the presence of ATP.

The fraction IV-generated recombinant rec50 exhibited a *Bgl* I cleavage pattern very different from that of the previously isolated rec22 (8) and p7-R5/R6 recombinants (6) (Fig. 2*b*), although the nucleotide sequences at the right sites of junctions in rec22, rec50, and p7-R5/R6 were identical (Figs. 2*c* and 5). The sites of recombination (open arrowheads) of several recombinants generated in the cell-free system are indicated in the sequences shown in Fig. 2*c*: p7-R5/R6, generated with fraction I (6); rec22 (8) and rec1(PCR), with fraction II; rec36, with fraction III′; rec50, with fraction IV (all this study). Recombinant rec3(PCR) arose by cell-free recombination with fraction IV (Fig. 2*c*). Its site of recombination was identical with that in rec18 (8), which was produced with fraction II. In some of the sequence determinations at the site of junction in rec3(PCR), the nucleotide sequence GCA was found to be repeated twice or three times (not shown) starting with nucleotide 1456 (Fig. 2*c*).

Proteins in fractions I, II, III′, and IV were targeted to very similar sites in the p7 DNA sequence and elicited independent cell-free recombination events. The target sequence in rec1(PCR) differed in only one nucleotide (C instead of T) from the frequently occurring target sequence 5′-CCTCTCCG-3′ (Fig. 2*c*). In recombinants rec18 and rec3(PCR), the target sequences were different from those in oligo 1.

Application of the PCR Method. The *E. coli* transfection assay could only detect recombination products that were infectious for *E. coli*. Moreover, it was difficult with this assay to exclude the possibility rigorously that *E. coli* might have contributed to the recombination process (6). We, therefore, adapted the PCR (12) to identification of recombinants produced in the cell-free system with fraction II or IV (Fig. 2). These recombinants were analyzed with synthetic oligo primers. Their locations were chosen so that a primer was unique for either the circular p7 plasmid (Fig. 4*a*, P1, P1′) or the linear *Pst* I D fragment of Ad12 DNA (Fig. 4*a*; A1, A2; A1′, A2′). The pBR322-cloned p7 sequence was used as circular construct, and its primers were located in the Tet^r of the vector and outside the p7 sequence, since it could not be predicted where in the p7 sequence recombination might

Table 1. Quantitative aspects of the purification of nuclear extracts

Fraction	Total protein in fraction, mg	% recovery of protein	No. of bacterial colonies	% positive colonies
I	15	100	—	—
II	4	26.6	16,650	0.12
III	2	13.3	1,800	0.11
IV	0.4	2.6	1,000	0.5

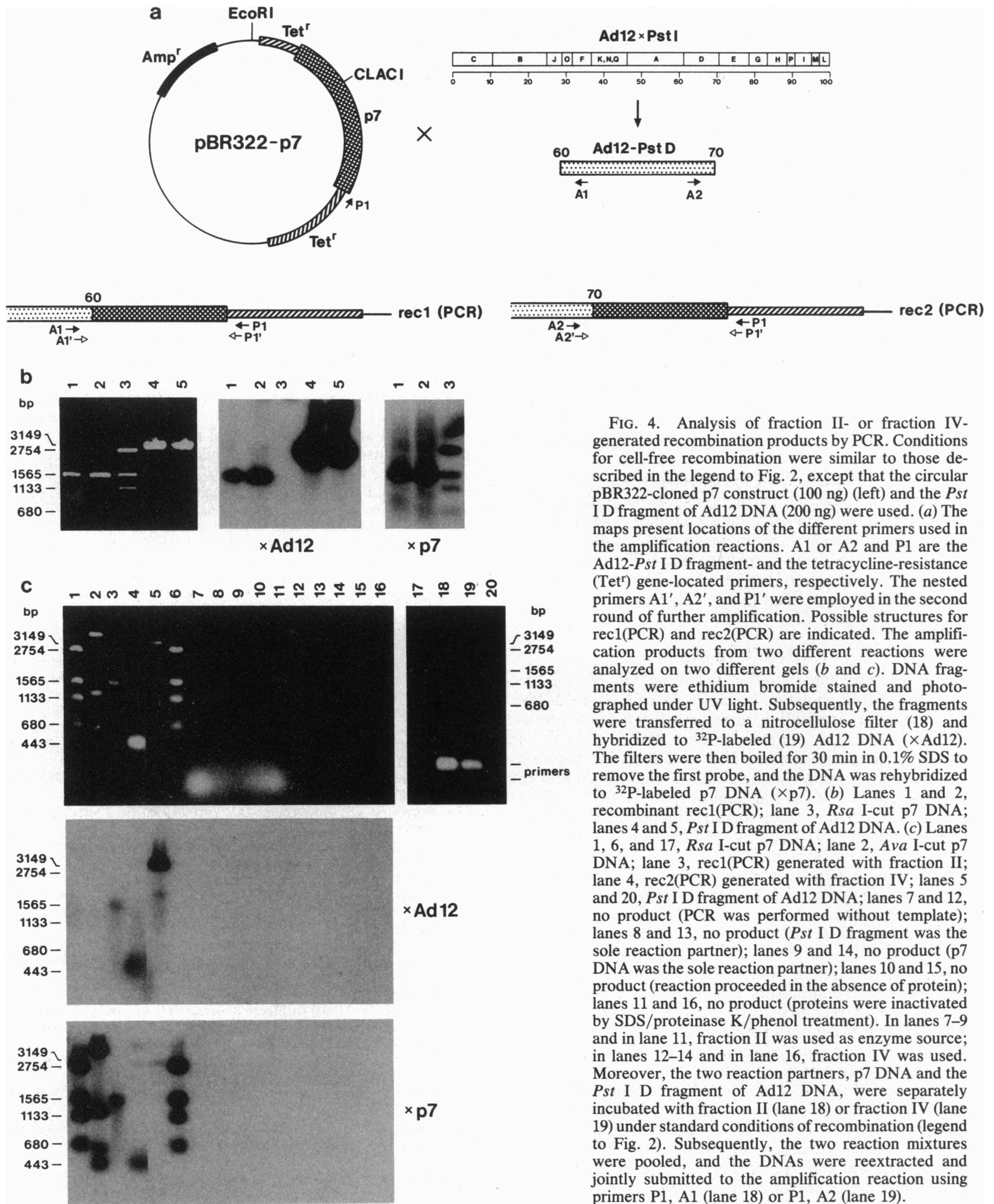

FIG. 4. Analysis of fraction II- or fraction IV-generated recombination products by PCR. Conditions for cell-free recombination were similar to those described in the legend to Fig. 2, except that the circular pBR322-cloned p7 construct (100 ng) (left) and the *Pst* I D fragment of Ad12 DNA (200 ng) were used. (*a*) The maps present locations of the different primers used in the amplification reactions. A1 or A2 and P1 are the Ad12-*Pst* I D fragment- and the tetracycline-resistance (Tet^r) gene-located primers, respectively. The nested primers A1′, A2′, and P1′ were employed in the second round of further amplification. Possible structures for rec1(PCR) and rec2(PCR) are indicated. The amplification products from two different reactions were analyzed on two different gels (*b* and *c*). DNA fragments were ethidium bromide stained and photographed under UV light. Subsequently, the fragments were transferred to a nitrocellulose filter (18) and hybridized to ^{32}P-labeled (19) Ad12 DNA (×Ad12). The filters were then boiled for 30 min in 0.1% SDS to remove the first probe, and the DNA was rehybridized to ^{32}P-labeled p7 DNA (×p7). (*b*) Lanes 1 and 2, recombinant rec1(PCR); lane 3, *Rsa* I-cut p7 DNA; lanes 4 and 5, *Pst* I D fragment of Ad12 DNA. (*c*) Lanes 1, 6, and 17, *Rsa* I-cut p7 DNA; lane 2, *Ava* I-cut p7 DNA; lane 3, rec1(PCR) generated with fraction II; lane 4, rec2(PCR) generated with fraction IV; lanes 5 and 20, *Pst* I D fragment of Ad12 DNA; lanes 7 and 12, no product (PCR was performed without template); lanes 8 and 13, no product (*Pst* I D fragment was the sole reaction partner); lanes 9 and 14, no product (p7 DNA was the sole reaction partner); lanes 10 and 15, no product (reaction proceeded in the absence of protein); lanes 11 and 16, no product (proteins were inactivated by SDS/proteinase K/phenol treatment). In lanes 7–9 and in lane 11, fraction II was used as enzyme source; in lanes 12–14 and in lane 16, fraction IV was used. Moreover, the two reaction partners, p7 DNA and the *Pst* I D fragment of Ad12 DNA, were separately incubated with fraction II (lane 18) or fraction IV (lane 19) under standard conditions of recombination (legend to Fig. 2). Subsequently, the two reaction mixtures were pooled, and the DNAs were reextracted and jointly submitted to the amplification reaction using primers P1, A1 (lane 18) or P1, A2 (lane 19).

occur. The *Pst* I D fragment of Ad12 DNA was excised from a plasmid devoid of the Tet^r sequence. After identification of amplified recombinants by Southern blot hybridization (18), they were further amplified by using primers (P1′, A1′, A2′ in Fig. 4*a*) at locations nested slightly internally to those previously employed. The amplification products of two different recombinants generated by fraction II [rec1(PCR)] (Fig. 4*b*) or IV [rec2(PCR)] (Fig. 4*c*) were shown. The length of these DNA fragments was 1600 bp (1800 bp in the first PCR) for rec1(PCR) or 450 bp (675 bp) for rec2(PCR). A third fraction IV-generated recombinant, rec3(PCR) (Fig. 2*c*), yielded amplification products of 740 bp (970 bp) (not shown in Fig. 4). The fragments hybridized to Ad12 DNA (×Ad12) and to p7 DNA (×p7) (Fig. 4 *b* and *c*). The results of several

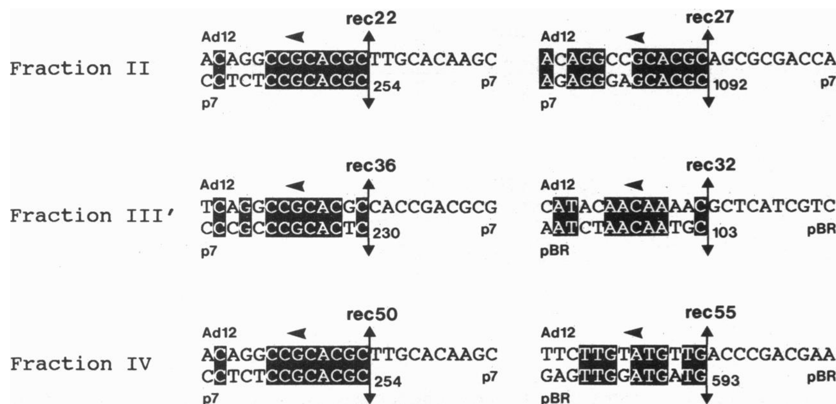

FIG. 5. Patch homologies at sites of cell-free recombination between p7 and Ad12 DNAs in different recombinants. The recombinants shown here were produced with the following protein fractions: rec22 and rec27, fraction II; rec32 and rec36, fraction III′; rec50 and rec55, fraction IV (see Fig. 1). All recombinants were identified by the transfection assay (6, 8). The sites of linkage between Ad12 DNA and p7 DNA or pBR322 DNA were designated by a double-headed arrow. The p7 or pBR322 nucleotide sequences that were replaced by Ad12 DNA were aligned under the Ad12 nucleotide sequence, and sequence identities are marked by dark background. Nucleotide numbers refer to published sequences (6, 20, 21). Arrowheads indicate the left to right (nucleotides 20,880–24,049; ref. 21) orientation of the sequence in the Ad12 genome.

control experiments (Fig. 4*c*, lanes 7–16, 18, and 19; for details see legend to Fig. 4*c*) validated the specificities of the amplification reactions. None of these control experiments led to the amplification of specific DNA fragments (Fig. 4*c*, lanes 7–16, 18, and 19). These negative results argued against the possibility of template switching by the Taq polymerase or other artifacts.

Sequence Characteristics of Recombinants. Patch homologies between the reaction partners play a role in the generation of recombinants in Ad12-induced tumor cells (3) and in cell-free recombination experiments (6, 8, 22). Patch homologies in the recombinants were illustrated in Fig. 5 for rec22, rec27, rec36, rec32, rec50, and rec55, which were generated by nuclear fractions purified to different extents (see legend to Fig. 5).

CONCLUSIONS

Cell-free systems have proved useful in studies on the mechanism of recombination in eukaryotic cells (6–8, 22–25). The consecutive application of several purification procedures has led to the enrichment of a limited number of proteins that facilitate the cell-free recombination between the hamster preinsertion sequence p7 (6) and the 20,880- to 24,049-nucleotide *Pst* I D fragment of Ad12 DNA (21). This reaction mimics elements of integrative recombination. In the most highly purified fraction IV, topoisomerase I and helicase could be demonstrated.

Cell-free recombinants in this system have been documented by two assays based on entirely different principles: (*i*) transfection into recA$^-$ strains of *E. coli* and isolation of recombinant plasmids and (*ii*) amplification of recombination products by PCR and direct sequence determination. The successful application of PCR to the identification of the *in vitro* generated recombinants proves that the hamster p7 DNA-Ad12 DNA recombinants have been derived from the cell-free system by *in vitro* recombination.

The summary of data presented in Fig. 2*c* suggests an interesting clustering of the sites of recombination in certain regions (e.g., oligo 1) of the 1768-bp hamster p7 DNA fragment. Of course, we have analyzed only a limited number of recombinants; this finding must, therefore, not be overinterpreted. Other sequences in the p7 segment have also served as acceptor sites [ref. 8; rec18 and rec3(PCR) in Fig. 2*c*]. The location of this clustering in the p7 sequence is, however, suggestive, as it encompasses the original preinsertion site of Ad12 DNA integration when the CLAC1 tumor was induced by Ad12 (9, 10).

It is significant that proteins in the fractions that catalyze cell-free recombination also bind specifically to the nucleotide sequence that constitutes oligo 1 (Fig. 2*a*; ref. 8). This binding is not inhibited by unrelated adenovirus DNA fragments or by poly(dA-dT)·poly(dA-dT) but, rather, by similar oligos from the p7 sequence that also carry the site of clustering of *in vitro* generated recombinants.

Results obtained in our laboratory with insect cell nuclear extracts (25) are reminiscent of those in the hamster cell system and argue for the general importance of the nonhomologous insertion reaction in eukaryotic cells.

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