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Nucleotide sequence analysis of the linked left and right hand terminal regions of adenovirus type 5 DNA present in the transformed rat cell line 5RK20

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ABSTRACT

A peculiar phenomenon is observed in several adenovirus type 2 or 5 (Ad2 or Ad5) transformed cell lines: the right hand and left hand terminal regions of the viral genome present in the viral DNA insertions of these cell lines are found to be linked together.
A large part of the viral DNA insertion present in the Ad5 transformed rat cell line 5RK20 has been cloned in the lambda vector Charon21A, including the segment containing the linked terminal regions. Sequence analysis of the linkage region showed a perfect homology with the Ad5 DNA sequence and a direct linkage of basepair (bp) 63 of the left hand end of the viral genome to bp 108 of the right hand end. No cellular or rearranged viral sequences were present. Our findings suggest that the joining of the terminal viral regions preceeded the integration of the viral sequences into the cellular genome.

INTRODUCTION

All cells transformed by Ad2 or Ad5 carry viral DNA sequences integrated into the genome of the host cell. The presence and expression of the left hand early region E1 of the viral genome is necessary for initiation and maintenance of transformation (1). No specific sites or sequences in either the host DNA or the viral DNA seem to be involved in integration (2-6). From the analysis of the integrated viral sequences in a fairly large number -15- of Ad2 or Ad5 transformed cell lines a peculiar phenomenon emerged (2-5). In several of these lines the integrated left and right hand terminal viral sequences appeared to be linked to each other. Detailed analysis by Southern blotting experiments of the rat cell lines 5RK20 (3) and 8617 (5) and the hamster cell lines HE2 and HE3 (4) shows a sequence arrangement in which the originally terminal segments of viral DNA have been inverted and linked to each other, either directly or via a short

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stretch of cellular DNA. A different arrangement of terminal regions is observed in the Ad2 transformed F4 cells (2). The linkage observed in the cell lines 5RK20, 8617, HE2 and HE3 could have occurred via a circular intermediate or via tandemly integrated viral genomes followed by deletion events.
In order to elucidate the exact nature of the observed linkage in 5RK20 cells we decided to clone and sequence the integrated viral sequences present in this cell line. This highly tumorigenic cell line resulted from the infection of primary rat kidney cells with UV-irradiated Ad5. It contains viral sequences from the left and right hand terminal regions, which are linked to cellular sequences somewhere between the map positions (m.p.) 11.6 and 17.0, and m.p. 69.0 and 72.8 respectively (Fig.1). The copy number of the viral sequences averages one copy per diploid cellular genome equivalent (3). Viral mRNAs and proteins of early regions E1, E3 and E4 have been observed in this cell line (7).
From detailed restriction mapping and sequence analysis it appeared that the viral termini have been directly linked to each other, accompanied by the loss of most sequences of the inverted terminal repeats. Apparently, no further rearrangements of viral sequences have taken place.

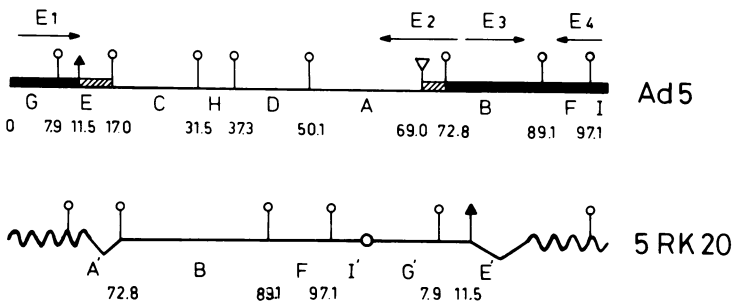

Fig. 1. Upper panel. Restriction enzyme map of Ad5 DNA. Viral DNA sequences present in 5RK20 cells are represented by the black bar, the presence of viral sequences corresponding to the shaded parts being uncertain. The symbols ⚲, ↑ and ⚲ stand for HindIII, SmaI and BglII sites resp. The figures beneath the bar are map units. The arrows above the bar show the position of the early regions in the Ad5 genome.
Lower panel. Arrangement of the integrated viral sequences. Wavy lines represent flanking cellular sequences, the dips in the solid lines correspond to sequences containing the host-viral junctions. The open circle locates the linkage of the viral termini.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from either New England Biolabs (Beverly, MA) or Boehringer (Mannheim). Calf intestine alkaline phosphatase, T4 polynucleotide kinase and E.coli DNA polymerase I were obtained from Boehringer. T4 DNA ligase was a gift from Dr. P. Weisbeek. Bacterial alkaline phosphatase was from Worthington (Freehold, N.J.). α-^{32}P-dCTP and γ-^{32}P-ATP (specific activity 2-3000 Ci/mmole for both) were purchased from the Radiochemical Centre (Amersham). Nitrocellulose filters were from Schleicher and Schüll (Dassel,Germany). Acrylamide and bis-acrylamide (purified for electrophoresis) were from BDH (Poole, England). Low melting point agarose was from Marine Colloids Inc. (Miles, England). All other materials used were reagent grade.

Preparation and screening of recombinant phages

All cloning procedures used were essentially carried out as described by Maniatis et al (8), unless stated otherwise. 5RK20 DNA was purified as described (9) and cleaved by HindIII. The digest was extracted with phenol and centrifuged through a 10-40% linear sucrose gradient. Fractions containing between 3 and 5 kilobase-pairs (kb) long fragments were pooled and used for cloning. The EK2 certified bacteriophage Charon21A (10) was used as a vector and propagated in the E.coli strain DP50supF. Charon21A DNA was purified, digested with HindIII and dephosphorylated with either calf intestine or bacterial alkaline phosphatase. The digest was incubated with ligase and afterwards tested for the absence of biological activity ($<10^5$ plaque-forming units/µg DNA). 5 µg digested Charon 21A DNA was ligated with 0.5 µg of enriched 5RK20 DNA fragments in a total reaction volume of 25 µl. In vitro packaging extracts were prepared and used according to a modification of the method of Hohn (11), using sonication and a freeze-thaw procedure to produce the lysates. The biological activity of the packaged recombinant DNA amounted more than 10^6 pfu/µg. Recombinant libraries were concentrated, amplified and screened as described before (6). Plaques giving positive signals were purified at least three times. Recombinant phages were propagated and purified as described before (6) and DNA was prepared according to Maniatis et al(8). All cloning experiments were performed

in the CI laboratories of the Institute of Molecular Biology in Utrecht.

Electrophoresis, blotting and hybridization procedures

Electrophoresis, Southern blotting, nick-translation and hybridization procedures have been described before (3). Restriction fragments of RK20I DNA were purified from a 0.7% low melting point agarose gel by the method of Wieslander (12).

All viral SmaI fragments used for hybridizations were cloned by Stenlund et al (13) and propagated in DP50supF.

Nucleotide sequence determination

The nucleotide sequence was determined by the method of Maxam and Gilbert (14), which was slightly modified according to Kruyer et al (15).

RESULTS

Cloning of DNA restriction fragments of the cell line 5RK20

5RK20 DNA was digested with restriction endonuclease HindIII and fragments of different size classes were separated by sucrose gradient centrifugation. Fractions containing predominantly fragments between 3 and 5 kb long were pooled and the DNA was ligated to Charon21A DNA, that had been digested with HindIII and dephosphorylated. The ligated DNA was packaged *in vitro* and the obtained library of 5RK20 DNA was amplified and screened for the presence of recombinant phages containing Ad5 DNA by the method of Benton and Davis (16).

Out of 4 x 10^5 recombinants tested, five clones hybridized with Ad5 DNA. Two of these clones also hybridized with both the terminal Ad2 SmaI fragments J and K (m.p. 0-2.8 and 98.3-100 resp.). In the course of this study these clones appeared to be identical and were both designated RK20I. Two of the three other clones (also identical and both designated RK20II) hybridized with the Ad2 SmaI-G fragment (m.p. 91.9-98.3), and the fifth clone (designated RK20III) with the Ad2 SmaI-A fragment (m.p. 56.9-75.8).

Restriction mapping of the viral DNA containing clones

Upon digestion of the recombinant DNA with HindIII and subsequent agarose gel electrophoresis, in clone RK20I DNA a restriction fragment could be detected comigrating with 5RK20 fragment no.3 (Fig.2), which fragment contains the terminal viral regions.

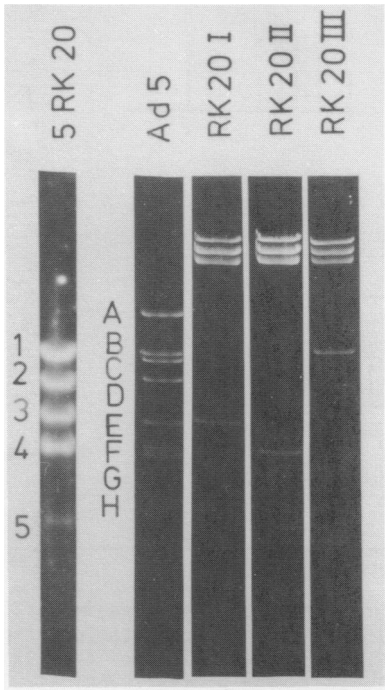

Fig. 2. Electrophoresis patterns of HindIII digests. The 5RK20 lane is a negative of an autoradiograph of blotted 5RK20 DNA, hybridized with ^{32}P-labeled Ad5 DNA. The other lanes are from gels stained with ethidium bromide.

Clone RK20II contained a HindIII fragment comigrating with 5RK20 fragment no.4 and the Ad5 HindIII-F fragment, while clone RK20III contained a HindIII fragment comigrating with 5RK20 fragment no.1 and the Ad5 HindIII-B fragment.

In addition, DNA of the three clones was digested with restriction endonucleases SmaI, XbaI, XhoI, HpaI, SacI, KpnI, BamHI, EcoRI and BglII respectively, and maps of the restriction sites were constructed (Fig.3). It appeared that the cleavage maps of the inserted HindIII fragments corresponded well with the cleavage map of Ad5 DNA, assuming that the HindIII fragments of clones III and II are identical to the Ad5 HindIII-B and -F fragments respectively and that clone RK20I contains the two terminal fragments linked together. The SmaI fragment spanning the linkage site was estimated to be 1400 basepairs long.

This SmaI fragment was purified by electrophoresis over a 0.7% low melting point agarose gel. After end-labeling by T4 polynu-

Fig. 3. Restriction maps of viral DNA sequences in 5RK20 cells present in the Charon21A recombinants. The figures indicate the corresponding viral map positions in map units. The open circle locates the linkage of the viral termini. The closed circle indicates a viral EcoRI site missing in the recombinant. The arrows indicate segments which were sequenced after 5'-end labeling.

cleotide kinase aliquots were digested with several restriction endonucleases (BalI, HhaI, TaqI, MspI, FnuDII, HphI and HaeIII). Subsequent electrophoresis of the digests over a 6% polyacrylamide gel followed by autoradiography of the gel showed the presence of all restriction enzyme sites present near the terminal SmaI cleavage sites of Ad5 DNA, except for the HphI cleavage site in the right hand terminal repeat (Fig.3).

Since the BalI site at bp 270 from the left hand viral terminus and the FnuDII site at bp 240 from the right hand viral terminus were shown to be present, we decided to determine the nucleotide sequence between these two cleavage sites, which were estimated to be separated by a stretch of 350 basepairs.

Another difference observed between the cleavage maps of the clonal DNA fragments and the corresponding Ad5 DNA fragments consisted of the absence of the EcoRI cleavage site at m.p. 75.9 from the RK20III clone. This site is also absent from the cellular 5RK20 DNA as was shown by Southern blotting analysis. De-

tailed restriction mapping and hybridization studies suggest that the absence of this site is due to either a very small rearrangement or a point mutation (data not shown). The disappearance of this site might be related to the fact that UV-irradiated virus has been used to obtain this transformed cell line.

Sequence analysis of the linkage between the viral termini

The SmaI fragment spanning the linkage site was digested with BalI, MspI and HhaI respectively, resulting fragments were end-labeled by T4 polynucleotide kinase, redigested with HaeIII and separated over a 6% polyacrylamide gel. The fragments carrying the linkage site were purified from the gel and the sequence of the fragments was determined from the labeled ends (for strategy see fig.3). The resulting sequence showed a perfect homology with the Ad5 DNA sequence between bp 262 and 63 of the left hand viral end (17) and bp 108 and 250 of the right hand viral end (18), the nucleotides corresponding to the left hand end bp 63 and the right hand end bp 108 being directly linked (Fig.4).

DISCUSSION

The restriction enzyme mapping, hybridization studies and sequence data obtained for the cloned DNA show that the cell line 5RK20 contains viral DNA sequences which are colinear with the Ad5 genome between m.p. 72.8-99.7 and m.p. 0.2-7.9. These viral sequences are fused together at the m.p. 99.7 and 0.2.

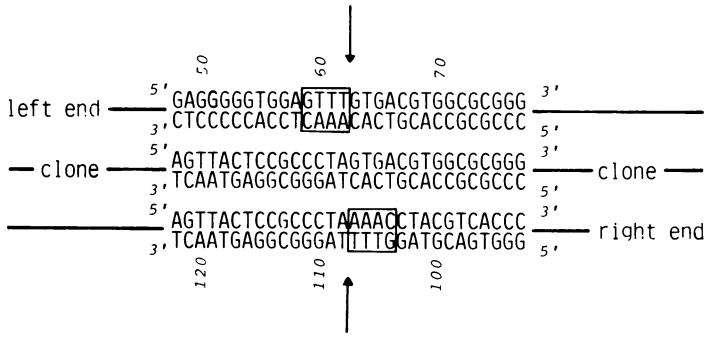

Fig. 4. The nucleotide sequence at the linkage site of the viral terminal regions in 5RK20 cells. The corresponding sequences, including the basepair numbers, of the left and right hand viral ends are shown above and below resp. The arrows show the exact position of the linkage. The boxes indicate homologous sequences in the left and right hand viral ends.

The high frequency of viral insertions in adenovirus transformed cells showing a similar type of linkage (4 out of 15) is remarkable and a model explaining the observed type of linkage should accommodate for this high frequency.

The nucleotide sequence at the site of linkage does not shed any light on the mechanism of recombination involved. Although short stretches of homology are present at the viral sites that have participated in recombination (see fig.4), it is unclear how these homologies could be functional with respect to the discussed recombination process. In the Ad5 transformed cell line 5RK20 bp 63 is linked to right hand terminal sequences, while in the Ad2 transformed cell line F4 it is the same GC-basepair that is linked to right hand terminal sequences (2,19). Whether this reflects the preferential involvement of a specific viral site in recombination, can only be deduced from the sequence arrangments of many more adenovirus transformed cell lines.

No cellular sequences occur at the site of linkage in the viral insertion of 5RK20. Although the experimental data do not rule out the possibility that the two viral ends have been inserted in close proximity of each other, followed by deletion of the intervening cellular sequences and some sequences of the viral terminal repeats, it is difficult to see which interactions might specifically promote such integration patterns and subsequent deletions.

Rather, the absence of cellular sequences at the site of linkage suggests that the joining of the viral terminal regions has preceeded their integration into the cellular genome. The adenovirus terminal protein, covalently bound to the 5'-ends of the viral DNA molecule, might promote such recombination of the terminal regions. By virtue of their mutual interactions these proteins are able to keep linear adenovirus DNA molecules in a circular state as appears from in vitro studies (20). In this circular state the ends of the viral DNA molecule are situated close together. This phenomenon may also occur in vivo, thus facilitating recombination between the terminal viral DNA regions. Furthermore, having a role in the initiation of replication (21), the terminal protein might exhibit affinity not only for its own kind, but also for specific sequences within the terminal viral

regions. Such a property could enhance recombination frequencies within these regions.

Linkage of left hand and right hand terminal regions also appears to occur in F4 cells. Although the actual arrangement of viral sequences in this cell line is quite different from the arrangement detected in 5RK20 cells, the terminal protein might have been similarly involved in the formation of a hypothetical intermediate arrangement as proposed by Sambrook et al (2).

In conclusion, the properties of the adenovirus terminal protein might well explain the high frequency of linked terminal viral regions encountered in adenovirus transformed cell lines. Whether the linked terminal regions have been derived from one viral molecule, which implies the formation of a circular intermediate, or from different molecules, can not be decided.

No sequences essential for transcription of either early region E1 or early region E4 appear to be deleted from the linkage site of the viral insertion of 5RK20 cells. In accordance with this, viral mRNAs and proteins corresponding to the E1 and E4 regions appear to be expressed (7). When compared with RNA synthesis observed early in lytic infections, RNA hybridizing with the right hand terminal Ad5 HindIII-I fragment was observed in relatively large amounts in nuclear, but not in cytoplasmic fractions. Since this DNA fragment only codes for the E4 promoter region and part of some late mRNAs which are never found to be expressed in transformed cells, read-trough products initiated but not properly terminated in the left hand l-strand might be resposible for the observed strong hybridization.

In order to complete our knowledge of the viral DNA insertion of this cell line, we presently attempt to isolate two additional recombinants containing the host-viral junctions of the insertion.

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