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Integration and Expression of Viral DNA in Cells Transformed by Host Range Mutants of Adenovirus Type 5

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Group I host range (hr) mutants of adenovirus type 5 are unable to transform rat embryo or rat embryo brain cells but induce an abnormal transformation of baby rat kidney cells. We established several transformed rat kidney cell lines and characterized them with respect to the transformed phenotype and the structure of the integrated viral DNA. The hr mutant-transformed cells, unlike wild-type virus transformants, were fibroblastic rather than epithelial, failed to grow in soft agar, and were also less tumorigenic in nude mice. Studies on the structure of the integrated viral DNA sequences showed that hr-transformed cells always contained the left end of the adenovirus DNA, but the size of the integrated DNA fragment varied among different lines, and a high percentage of the lines contained the entire viral genome colinearly integrated. The patterns of integration were maintained after prolonged growth in culture and after subcloning. Attempts to rescue infectious virus from lines which contained the entire genome were unsuccessful. Using immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, we analyzed the viral proteins expressed in hr-transformed cells. Results of these studies indicated that, like wild type-transformed cells, hr transformants expressed E1B proteins of molecular weight 58,000 and 19,000.

During lytic infection of permissive cells by human adenovirus type 2 or type 5 (Ad2 or Ad5), at least four to five separate regions of the viral genome are expressed early (before DNA replication) (3, 6, 12, 27). Of these, early region 1 (E1), contained within the left 12% of the viral genome, has been most clearly implicated in cell transformation (11, 15, 37, 41, 44). In addition to E1, at least two other early regions may play some role in transformation of cells by virus (though not by purified viral DNA). These are the E2B region (12, 40), in which the transformation-defective mutants *ts*36 and *ts*149 have been mapped (12, 46, 47), and the E2A region, where *ts*125 and *ts*107 are located (18, 42) and which may share a common promotor with E2B (40). Results of studies with temperature-sensitive mutants (46, 47) have suggested that the E2B functions are required for initiation of transformation by virus, but are dispensable once the transformed state is established, a hypothesis consistent with the fact that many apparently fully transformed cells do not contain the E2B region and that cells can be transformed by DNA fragments containing only sequences to the left of E2B. The role of E2A in transformation is somewhat obscure, since the E2A mutant *ts*125 has been shown to transform with enhanced efficiency at both the permissive and nonpermissive temperatures (13). Again, E2A functions presumably are not required for maintenance of transformation.

If any viral products are required for maintenance of the transformed phenotype, these products must be coded within E1. This region can be subdivided into two transcriptional units: E1A, from 1.3 to 4.4 map units, and E1B, from 4.6 to 11.1 map units (3, 6). A number of host range (hr) mutants of Ad5 have been isolated on the basis of their preferential growth in an adenovirus-transformed human cell line containing and expressing the left 12% of the adenovirus DNA (19). These mutants are transformation defective (16) and appear to fall into two complementation groups, I and II, mapping in E1A and E1B, respectively (10). The hr mutants of group I are unable to transform rat embryo or rat embryo brain cells but induce an abnormal or semiabortive transformation of baby rat kidney (BRK) cells (16). Group II hr mutants, on the other hand, fail to transform any of these cell types in transformation assays using virus (16).

Lines of BRK cells transformed by hr mutants of group I have been established, and preliminary results on their characterization have been reported (16). In the studies described in this paper, we established additional lines and characterized them in terms of extent and structure of integrated viral DNA, expression of virus-specific proteins, a variety of growth parame-

ters, and tumorigenicity. The results are consistent with the hypothesis that group I hr mutants are defective in a function required for the induction and maintenance of a fully transformed phenotype. In addition, the structure of integrated viral DNA in several hr mutant-transformed cells appears to be atypical.

MATERIALS AND METHODS

Cells and virus. The methods used to grow HeLa and 293 cells and to grow, purify, and assay wild-type and mutant virus, as well as the virus strains used, have been described (16, 17, 19). For transformation experiments, primary BRK cells were prepared from 1-week-old Wistar rats and seeded in α-minimal essential medium supplemented with 10% fetal calf serum. Transformed cells were grown in Joklik medium with 10% fetal calf serum or newborn calf serum.

Transformation assays. One- to two-day-old subconfluent cultures of primary BRK cells (approximately 10^6 cells/60-mm dish) were infected with wild-type virus or hr mutant virus at a multiplicity of infection of 0.01 to 2 PFU/cell. The hr mutants 1, 3, and 5 from group I and 6 from group II were used. After 1 h of absorption at 37°C, the cultures were refed with α-minimal essential medium plus 10% fetal calf serum and 3 days later switched to Joklik modified minimal essential medium (with low Ca^{2+}) plus 5% horse serum. This medium was changed two or three times per week. Dishes in which colonies could be detected were transferred as mass cultures at different times after infection. Once established, some of the lines were subcloned by plating 20 to 50 cells/60-mm dish and picking no more than one clone from each dish.

Growth and plating efficiency studies. Cell saturation densities and doubling times were measured in cultures growing in Joklik medium with 10% fetal calf serum. Cells were seeded in 60-mm dishes at 2×10^5 cells/dish; each day over a 10-day period, a set of cultures was trypsinized, and the cells were counted in a Neubauer chamber. The medium was changed once in the middle of the experiment (at about 5 days). The doubling time of the cells was calculated from the exponential part of the growth curve and the saturation density, expressed as number of cells per 60-mm dish, was calculated from the plateau level reached in each case. The efficiency of plating in soft agar was determined according to Macpherson and Montagnier (28) by plating 10^3 to 10^5 cells in 2 ml of medium containing 0.25% Noble agar (Difco Laboratories) onto a basal layer of 0.5% agar. Colonies were counted after 6 to 8 weeks with the aid of a low-power microscope.

Tumorigenicity in nude mice. Confluent cultures were trypsinized, and the cells were suspended in phosphate-buffered saline (PBS) with 2% horse serum. After counting, the cells were centrifuged and suspended in PBS without serum, at a concentration of 0.8×10^8 to 2×10^8 cells/ml, and 0.1 ml of the cell suspension was inoculated subcutaneously into 6- to 8-week-old congenitally athymic nude mice (31).

DNA extraction. Confluent monolayers of transformed cells were rinsed twice with PBS lacking Ca^{2+} and Mg^{2+}, and the cells were lysed in 2 to 3 ml of 10 mM Tris-hydrochloride, pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.4% sodium dodecyl sulfate (SDS) plus 0.5 mg of predigested pronase B per ml (Calbiochem-Behring Corp.). After digestion of cell proteins at 37°C overnight and extraction with Tris buffer-saturated phenol and chloroform-isoamyl alcohol (24:1), the lysate was treated with 10 μg of RNase A per ml (Sigma Chemical Co.) for 30 min at 37°C and again extracted with chloroform-isoamyl alcohol. After dialysis against 10 mM Tris-hydrochloride, pH 7.5, 10 mM NaCl, and 1 mM EDTA, the DNA was precipitated with ethanol and dissolved in 20 mM Tris-hydrochloride, pH 7.5, and 1 mM EDTA. The DNA extraction from purified virions was carried out similarly except that digestion with pronase was for 2 h, and RNase treatment was omitted. DNA from recombinant plasmids containing the cloned fragment *Hin*dIII-F or *Xho*I-C, or the *Pvu*II leftmost 1.2% fragment (43) of Ad5 DNA, was provided by R. McKinnon (McMaster University).

In vitro labeling of DNA. Adenovirus DNA or cloned viral DNA fragments were labeled in vitro with [α-^{32}P]dCTP by nick translation essentially according to Maniatis et al. (29) or Rigby et al. (33). Specific activities of 0.8×10^8 to 1.8×10^8 cpm/μg of DNA were obtained.

Gel electrophoresis and Southern blotting of cell DNA. DNA from control or transformed cells was digested with various restriction endonucleases according to the manufacturer's instructions (New England Biolabs or Bethesda Research Laboratories). Thirty micrograms of each DNA digest was then electrophoresed on 0.5 or 0.7% agarose horizontal slab gels (16 by 26 by 1 cm) for about 36 h at 1 V/cm; one or three genome equivalents of Ad5 DNA per cell, mixed with 30 μg of salmon sperm DNA and digested in the same way as test DNA, was also electrophoresed as a marker. Transfer of the DNA from the gels to nitrocellulose filters (Schleicher & Schuell BA 85 R 597) was done by the Southern technique (38) as modified by Ketner and Kelly (23).

Hybridization and autoradiography. DNA-DNA hybridization was performed at 65°C in Denhardt solution (7) according to Botchan et al. (4) or at 42°C in the presence of formamide and dextran sulfate according to Wahl and Stark (45). Autoradiography was on Kodak X-Omat R films XR1 or XR5 with intensifying screens.

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Cultures of transformed cells at ~80% confluency were labeled for 8 to 12 h with 10 μCi of [^{35}S]methionine per ml in α-minimal essential medium lacking methionine. To detect Ad5 antigens, two sera were used: megaserum, provided by D. T. Rowe (McMaster University), and P antiserum, supplied by W. C. Russell (National Institute for Medical Research, Mill Hill, London). Megaserum is pooled serum from hamsters bearing tumors as a result of injection of several different lines of hamster cells transformed by the *Hin*dIII fragment G or *Xho*I fragment C of Ad5 DNA. This antiserum precipitates E1A and E1B proteins from virus-infected KB cells (D. T. Rowe and F. L. Graham, unpublished data). P antiserum was obtained from rabbits injected with extracts of Ad5-infected 1-β-D-arabinofuranosylcytosine-treated RK_3 cells (35). It is directed mainly against the 72,000-dalton single-stranded DNA-binding protein, but it is

also active against other adenovirus early polypeptides (25, 36). The protein A-Sepharose technique was used as described (25). Proteins from the immune precipitates were analyzed by SDS-polyacrylamide slab gel electrophoresis (25). The gels were dried under vacuum and autoradiographed on Kodak X-Omat film XR1 or XR5, using intensifying screens.

RESULTS

Isolation of cell lines transformed by hr mutant virus. Subconfluent monolayers of primary BRK cells were infected with wild-type Ad5, with group I hr mutant 1, 3, or 5, or with group II hr mutant 6 at a multiplicity of infection of 0.01 to 2 PFU/cell. Three days after infection, the cultures were switched to low-calcium medium (Joklik) plus 5% horse serum to select against untransformed rat cells. Extensive cytopathic effect was observed by 2 to 3 days postinfection in cultures infected with wild-type virus and, to a lesser degree, in group II mutant-infected cultures; no cytopathic effect was observed after infection with group I mutant virus. Transformed colonies were detected 10 to 14 days after infection in cultures inoculated with either wild-type or group I hr mutant virus, whereas no transformed foci developed in cultures infected with group II mutant virus, as reported previously (16). In agreement with results published by Graham et al. (16), the number of foci in cultures receiving group I mutant virus was higher than in cultures infected with wild-type virus. Attempts to isolate transformed clones from the mutant-infected cultures were unsuccessful; however, polyclonal transformed lines could be established by passaging entire cultures containing 20 or more colonies. Transformed cultures were trypsinized at various times ranging from 2 to 11 weeks postinfection and reseeded into 25-cm^2 plastic bottles in Joklik medium plus 5% horse serum. From a total of 48 attempts, 21 cultures continued to grow after the second or third passage and indefinitely thereafter. Table 1 presents the protocol for the isolation of the various lines; hr virus-transformed lines 637-1, 637-3, 637-4, and 809-1 and wild type-transformed lines 637-C3 and 822-C2 (both clonal isolates) were derived from previous experiments (16). The hr mutant-transformed lines had a fibroblastic morphology (Fig. 1B) different from the epithelial-like morphology of the wild-type transformants (Fig. 1A). Small groups of cells with epithelial morphology could be observed in some hr-transformed cultures at early passages (Fig. 1C) but disappeared after 8 to 12 passages. Clonal isolates could be obtained from the fibroblastic cells after eight to nine passages in culture, but attempts to clone the epithelial cells from hr-transformed cultures were unsuccessful.

Growth characteristics and tumorigenicity of transformed cells. Transformed lines, as yet uncloned, were initially characterized on the basis of several growth parameters. Both mutant- and wild-type transformants were capable of growth in low-calcium ion medium and have so far been passaged for over 50 times in culture. Initially, mutant-transformed lines grew slower than wild-type transformants, but after six to eight passages in culture both types of transformants had similar doubling times and saturation densities (data not shown). Cells transformed by hr mutants, however, did not form colonies in soft agar, whereas wild type-transformed cells usually plated with an efficiency of 10 to 15% (data not shown). Several transformed lines were injected into 6- to 8-week-old nude mice at concentrations ranging from 10^5 to 10^7 cells per animal. The hr-transformed cell lines were much less tumorigenic than lines transformed by wild-type Ad5 (Table 2). In fact, only one hr transformant, 637.1, induced any tumors in nude mice, and in this case the latent period (16 weeks) was much longer than that for most wild type-transformed lines, and only two of four animals developed tumors.

Viral DNA sequences in transformed cells. As described in the previous section, wild type- and hr-transformed cells exhibited significantly different phenotypes which could conceivably result from differences in integrated viral sequences or differences in the expression of viral genes. To explore these possibilities, the presence and state of viral DNA in hr-transformed lines was studied and compared with that in wild-type transformants. DNA from transformed cells was digested with *Xba*I (Fig. 2A) or *Hin*dIII (Fig. 2B), and the digestion products were separated by electrophoresis, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled Ad5 DNA. The cleavage maps of the viral DNA with either enzyme are shown above each panel of Fig. 2, and the migration patterns of the respective cleavage products of viral DNA markers are illustrated in track 6 of panel A (*Xba*I) and track 6 of panel B (*Hin*dIII). Comparison of these patterns with those obtained by electrophoresis of restricted DNA from transformed cells indicated that hr-transformed lines 1H2, 637-4, 5H1, and 5H5 (Fig. 2A, tracks 2, 7, and 8; Fig. 2B, tracks 15, 3, 7, and 2) contained all of the internal fragments of Ad5 DNA (i.e., *Xba*I fragments A, B, and D and *Hin*dIII fragments A, B, C, D, E, F, and H). Although the terminal fragments of viral DNA (*Xba*I fragments C and E or *Hin*dIII fragments G and I) could not be directly identified in the transformed cell DNA, all of the above lines contained two or more new fragments migrating slower than the DNA termini. The new frag-

TABLE 1. Cell lines transformed by group I hr mutant and wild-type virus[a]

Expt	Cell line	Transformed by:	Transformed at MOI[b] of:	Day of 1st subculturing[c]	DNA extracted at passage no.[d]
1	637-4	hr 1	5×10^{-2}	19	13, 41
	809-1	hr 1	2	28	8
	637-1	hr 3	1	19	11
	637-3	hr 3	2×10^{-1}	19	15
	637C3	wt	5×10^{-1}	19	32
	822C2	wt	2	21	12
2	1H1	hr 1	10^{-1}	32	4–5
	1H2	hr 1	10^{-1}	34	5, 11–13
	3H1	hr 3	1	13	5–6, 9–11
	3H2	hr 3	1	34	4–5
	5H1	hr 5	1	13	6–8, 9–10
	5H2	hr 5	1	19	6, 8–10
	5H3	hr 5	1	34	5, 8–11
	5H4	hr 5	10^{-1}	77	10–12
	W86	wt	1	47	11–13
3	1H3	hr 1	1	15	5–7
	3H3	hr 3	1	15	9–16
	5H5	hr 5	1	15	7–12

[a] Primary BRK cells were transformed by wild-type (wt) virus or by hr mutant 1, 3, or 5 as described in the text, and at various times postinfection polyclonal populations of transformed cells were trypsinized. Listed in the table are all of the established cell lines which are studied in the following sections.

[b] Multiplicity of infection (PFU/cell) at which the rat cells were infected.

[c] Number of days postinfection at which the first passage of the entire culture was done or at which two wild-type clones were isolated (637C3 and 822C2).

[d] Number of passages at which the cell DNA was extracted and studied.

ments most likely represent joint fragments between viral and host DNA sequences; if so, the patterns described above are consistent with the hypothesis that lines 1H2, 637-4, 5H1, and 5H5 contain all of the viral genome colinearly integrated.

As already mentioned, these hr-transformed lines were not clonal isolates, and therefore it seemed possible that the apparent integration of the entire genome could be due to a mixed population of cells carrying different regions of the viral DNA. This hypothesis, however, could be discounted when the DNA content of subclonal lines isolated from one hr-transformed line, 637-4, was analyzed. As shown in Fig. 3A, for DNA restricted with *Xba*I, all of the sub-

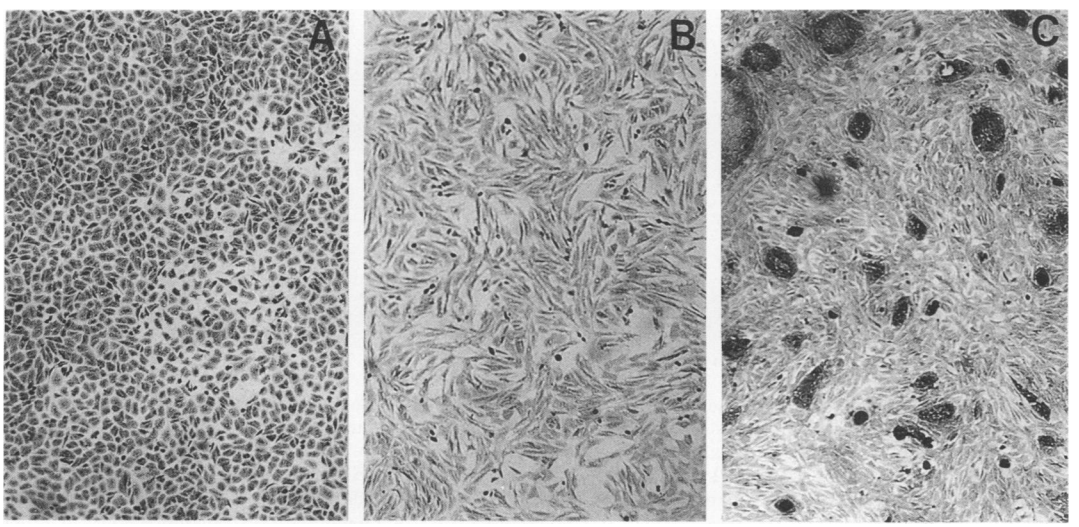

FIG. 1. Micrographs of transformed cells stained with May Grünwald-Giemsa (magnification, ×50). (A) Wild-type Ad5-transformed cells (W86, passage 21); (B) hr 1-transformed cells (1H2, passage 20); (C) hr 3-transformed cells (3H3, passage 5).

TABLE 2. Tumorigenicity of Ad5-transformed cell lines in adult athymic nude mice[a]

Cell line	Transformed by:	No. cells inoculated	No. with tumors/no. inoculated		
			Expt 1	Expt 2	Expt 3
637C3	wt[b]	10^7	5/5 (3)[c]	4/4 (3)[c]	4/4 (1)[c]
		10^6	4/4 (3)		
		10^5	3/4 (3)		
424C1	wt	10^7	4/4 (2)	4/4 (7)	4/4 (2)
	(sheared DNA)	10^6	3/3 (7)		
642C1	wt	10^7	4/4 (2)		
822C2	wt	10^7	0/4 (13)	0/4 (25)	
W86	wt	10^7	1/4 (4)		
1H2	hr 1	10^7	0/4 (8)	0/4 (5)	
1H3	hr 1	10^7	0/4 (9)		
637-4	hr 1	$10^{7,6,5}$	0/4 (26)	0/4 (16)	
3H3	hr 3	10^7	0/4 (10)		
637-1	hr 3	10^7	2/4 (16)	0/4 (14)	
5H2	hr 5	10^7	0/4 (8)		

[a] Transformed rat cells growing in culture were harvested, injected subcutaneously into nude mice, and scored for tumorigenicity as described in Materials and Methods.

[b] wt, Wild type.

[c] Latent period in weeks or time at which the experiment was terminated.

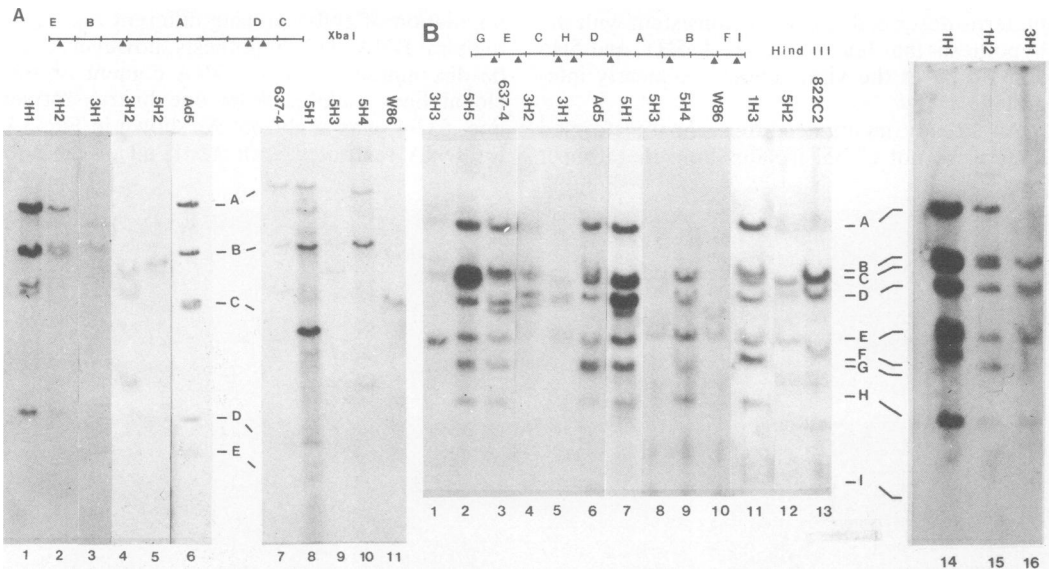

FIG. 2. Analysis of Ad5 DNA sequences present in hr- and wild type-transformed cells. DNAs extracted from transformed cells were digested with *Xba*I (A) or *Hin*dIII (B) and analyzed by Southern's blotting procedure (38) followed by hybridization, using ^{32}P-labeled Ad5 DNA as probe. The cell DNAs electrophoresed were as follows: (A) 1H1, channel 1; 1H2, channel 2; 3H1, channel 3; 3H2, channel 4; 5H2, channel 5; 637-4, channel 7; 5H1, channel 8; 5H3, channel 9; 5H4, channel 10; and W86, channel 11. *Xba*I-digested Ad5 DNA was run in channel 6 as a marker. (B) 3H3, channel 1; 5H5, channel 2; 637-4, channel 3; 3H2, channel 4; 3H1, channel 5; 5H1, channel 7; 5H3, channel 8; 5H4, channel 9; W86, channel 10; 1H3, channel 11; 5H2, channel 12; 822C2, channel 13; 1H1, channel 14; 1H2, channel 15; and 3H1, channel 16. *Hin*dIII-digested Ad5 DNA was run in channel 6 as a marker.

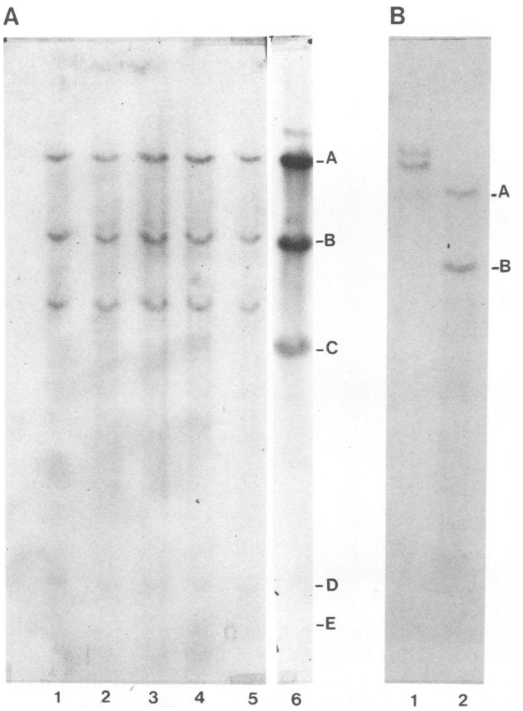

FIG. 3. Integration patterns of Ad5 DNA in the hr-transformed cell line 637-4 and its subclones. (A) Cell DNA extracted at passage 13 from line 637-4 (channel 1) or from four subclones isolated at passage 13 (channels 2 to 5) was digested with *Xba*I and hybridized to ^{32}P-labeled Ad5 DNA as described in the text. Ad5 DNA marker was electrophoresed in channel 6. (B) *Bam*HI-digested 637-4 (channel 1) and *Bam*HI-digested Ad5 DNA (channel 2).

clones derived from line 637-4 (tracks 2 to 5) exhibited the same integration pattern of Ad5 DNA as the parental line (track 1); this pattern, moreover, did not vary over a period of several months in culture (data not shown). The results, which are in agreement with the hypothesis that the entire viral genome is colinearly integrated in the transformed cells, were further confirmed by analysis of cell DNA restricted with *Bam*HI, which cleaves Ad5 DNA at a single site (at 59.5%) generating only two fragments. DNA from line 637-4 contained only two fragments complementary to the labeled probe (Fig. 3B, track 1), both of which migrated slower than the *Bam*HI A and B fragments of viral DNA (track 2), as expected if the viral genome is integrated at a single site into host DNA. Subclones from other cell lines were not analyzed by Southern blotting. However, in most cases the lines contained a single integration site for viral DNA and therefore were probably monoclonal. Where more than one site was found, as in the case of lines 3H2, 3H3, and 5H1 (see Fig. 5), it is possible that the lines contained a mixed population of two or more cell types.

The results of hybridization with probe made from total Ad5 DNA indicated that the hr-transformed lines contained widely differing lengths of viral DNA. Based on the presence of fragments comigrating with internal fragments of viral DNA, upon digestion with two restriction endonucleases, and assuming that all transformed cells must contain at least the left-end portion of the viral genome (11, 37, 44), the extent of viral DNA in the cells was estimated at between less than 17% up to as much as the entire genome. On the other hand, all of the wild type-transformed cell lines which were analyzed contained only a portion of the viral DNA, amounting to less than the leftmost 28% of the genome (see Fig. 2A, track 11, and 2B, tracks 10 and 13). To obtain more precise data on the viral DNA sequences present in each transformed line, particularly with respect to sequences near the viral termini, additional studies were performed, using as labeled probes *Xho*I-C (0 to 15.5%) or *Hin*dIII-F (89.1 to 97.1%) of Ad5 DNA cloned in pBR322. Figure 4A shows the results obtained when the *Xho*I-C probe was hybridized to cell DNA (tracks 2 to 12) or viral DNA (track 13) restricted with *Xba*I. In the case of Ad5 DNA, as expected, the probe hybridized to *Xba*I-E (0 to 3.8%) and -B (3.8 to 28.8%). Two or more hybridization fragments were also detected in the cellular DNAs. Some of the lines (1H1, 1H2, 3H1, 637-4, 5H3, 5H4; tracks 2 to 4, 6, 8, and 9) contained a fragment comigrating with *Xba*I-B and an additional fragment slower than *Xba*I-E which was assumed to be the viral left end-host junction fragment. Other lines did not contain any fragments of native size, but rather what was assumed to be the *Xba*I-E–host DNA junction and a subset of *Xba*I-B, again in the form of a joint fragment (lines 3H2, W86, 822C2, and 637C3; tracks 5 and 10 to 12).

Finally, some lines (3H2 and 5H1; Fig. 4A, tracks 5 and 7) contained more than two fragments hybridizing to the *Xho*I-C probe, which suggests more than one site of integration for the viral DNA. Similar conclusions were derived from the results obtained with cell DNA digested with *Hin*dIII and hybridized with the *Xho*I-C probe (data not shown). Additional experiments using the cloned extreme left-end 1.2% of Ad5 DNA (*Pvu*II left-end fragment, 454 base pairs) to probe DNA from lines 637-4, 1H2, and 1H3 showed a single hybridization band with the same mobility as one hybridizing to the *Xho*I-C probe (not shown). This result allows us to conclude that in these lines the virus-host junction on the left end of Ad5 DNA deletes less than 454 base pairs of the viral sequences.

In contrast to the results obtained by probing

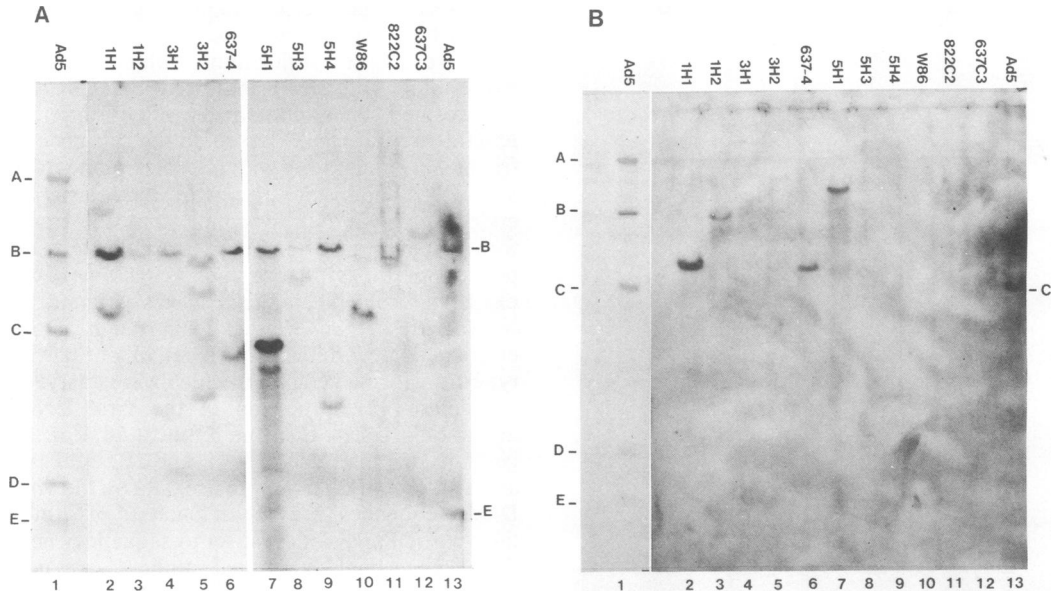

FIG. 4. Analysis of Ad5 DNA present in hr- and wild type-transformed cell lines. Filters prepared as described in Fig. 2 were hybridized with ^{32}P-labeled plasmid DNA containing the *Xho*I fragment C (A) or the *Hin*dIII fragment F (B) of Ad5 DNA. In both panels, the cell and virus marker DNA was digested with *Xba*I. Both panels illustrate, from left to right, DNA from lines: 1H1 (channel 2), 1H2 (channel 3), 3H1 (channel 4), 3H2 (channel 5), 637-4 (channel 6), 5H1 (channel 7), 5H3 (channel 8), 5H4 (channel 9), W86 (channel 10), 822C2 (channel 11), and 637C3 (channel 12). Channel 1 contains ^{14}C in vivo-labeled Ad5 DNA, and channel 13 contains cold Ad5 DNA hybridized with the same ^{32}P probe as the cell DNAs.

with the cloned left end of viral DNA (*Xho*I-C), the right end of the viral genome was absent from several lines (Fig. 4B). Hybridization with the cloned *Hin*dIII fragment F (89.1 to 97.1%) gave positive results only for lines which, from the results shown in Fig. 2 and 3, appeared to contain the entire genome. In these cases (Fig. 4B, tracks 2, 3, 6, and 7) one band, migrating slower than the marker *Xba*I fragment C (84.3 to 100%, track 13) could be detected.

The results presented in Fig. 2 to 4, as well as additional unpublished data, were used to construct the diagram in Fig. 5 showing the simplest and most probable integration pattern of Ad5 DNA in 16 different transformed lines.

Virus rescue experiments. The inability to rescue infectious virus from cells transformed by adenoviruses of group C has been attributed to the fact that these cells usually contain only a fraction of the viral DNA (9, 37). Some of the hr-transformed lines described here, on the other hand, appear to contain the entire genome and as such are suitable candidates for virus rescue attempts. For these experiments two procedures, successfully used in the papovavirus system (5, 32), were followed, using line 637-4. In one case, high-molecular-weight DNA from the hr-transformed cells was used to infect 293 cells (5), using the calcium technique (14); in the second case, hr-transformed and 293 cells were fused with the aid of polyethylene glycol (32). The 293 cells were chosen as recipients for the transformed cell DNA or as partner in the fusion experiments, since they are fully permissive for hr mutant virus (19). With either procedure, however, no infectious virus could be rescued. Several reasons can account for this failure: first, the integrated sequences may not extend all the way to the extreme ends of the viral DNA; second, excision of viral DNA may not occur or products required for viral DNA replication may not be produced after fusion or transfection; and finally, the original transformation may have been caused by virus particles with an additional defect besides the hr mutation.

Expression of viral proteins in hr-transformed cells. A number of workers have reported that deletion mutants and hr mutants mapping in E1A (22) are unable to transcribe other early regions, including E1B, in nonpermissive HeLa or KB cells (1, 2, 21). We considered it of interest, therefore, to examine the expression of early viral proteins in hr-transformed cells. Extracts of cells labeled with [^{35}S]methionine were immunoprecipitated with either Mega-

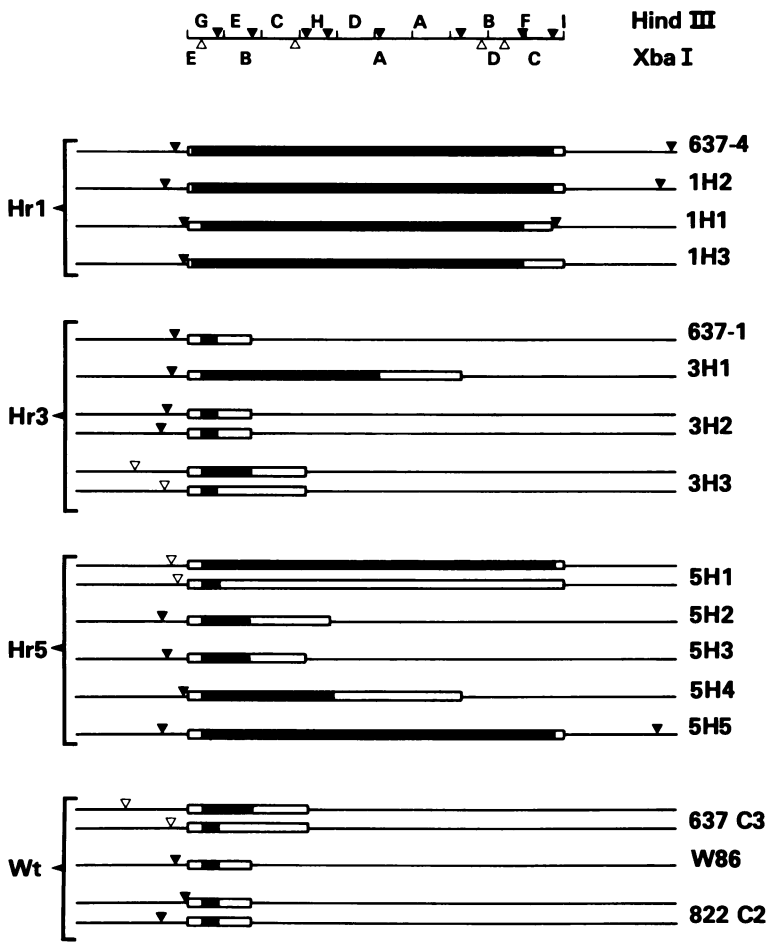

FIG. 5. Schematic representation of the integration patterns of Ad5 DNA in transformed rat cells. Only the most likely model is presented. Symbols: —, Cell DNA; ▼, *Hin*dIII restriction sites in the cell DNA; ■, virus DNA sequences present in the cells; □, virus DNA fragments for which the fraction present in the cells is uncertain. Lines 3H2, 3H3, 5H1, 637C3, and 822C2 have two distinct inserts of viral DNA (cf. Fig. 4). Because of the complexity of the pattern obtained for cell lines 3H3, 5H1, and 637C3, it is not possible to say which left-end junction corresponds to which insert, and the resulting uncertainty in the host cell DNA cleavage site is indicated in the figure by open triangles. The integration pattern after digestion with *Kpn*I and *Bam*HI (not shown) is consistent with the scheme illustrated above and does not reveal the existence of new integrated fragments. The right-end host DNA cleavage sites, when known, are included for cells which appear to contain the entire genome.

serum or P antiserum (see Materials and Methods), and the reaction products were electrophoresed on polyacrylamide gels (Fig. 6). P antiserum (Fig. 6A) was used to detect the expression of the 72,000-dalton and the related 67,000-dalton proteins in two transformed lines containing the E2 region of adenovirus DNA (i.e., 637-4 and 1H2). None of these transformed lines expressed either the 72,000- or the 67,000-dalton proteins (Fig. 6A, tracks 4 and 8). However, they expressed a polypeptide which migrated in the region of 58,000 daltons as a broad band. This protein was also detected in immunoprecipitates using Megaserum which is specific for El proteins (Fig 6A, tracks 3 and 7 for lines 1H2 and 637-4; Fig. 6B, tracks 3 and 5 for lines 5H2 and 637-C3).

In addition to the 58,000-dalton protein, a polypeptide of molecular weight 19,000 was also detected in every cell line when immunoprecipitation was carried out using Megaserum. These two polypeptides are thought to be coded by sequences in E1B (34; Rowe and Graham, unpublished data).

Both P antiserum and Megaserum are capable of precipitating E1A proteins from infected cells

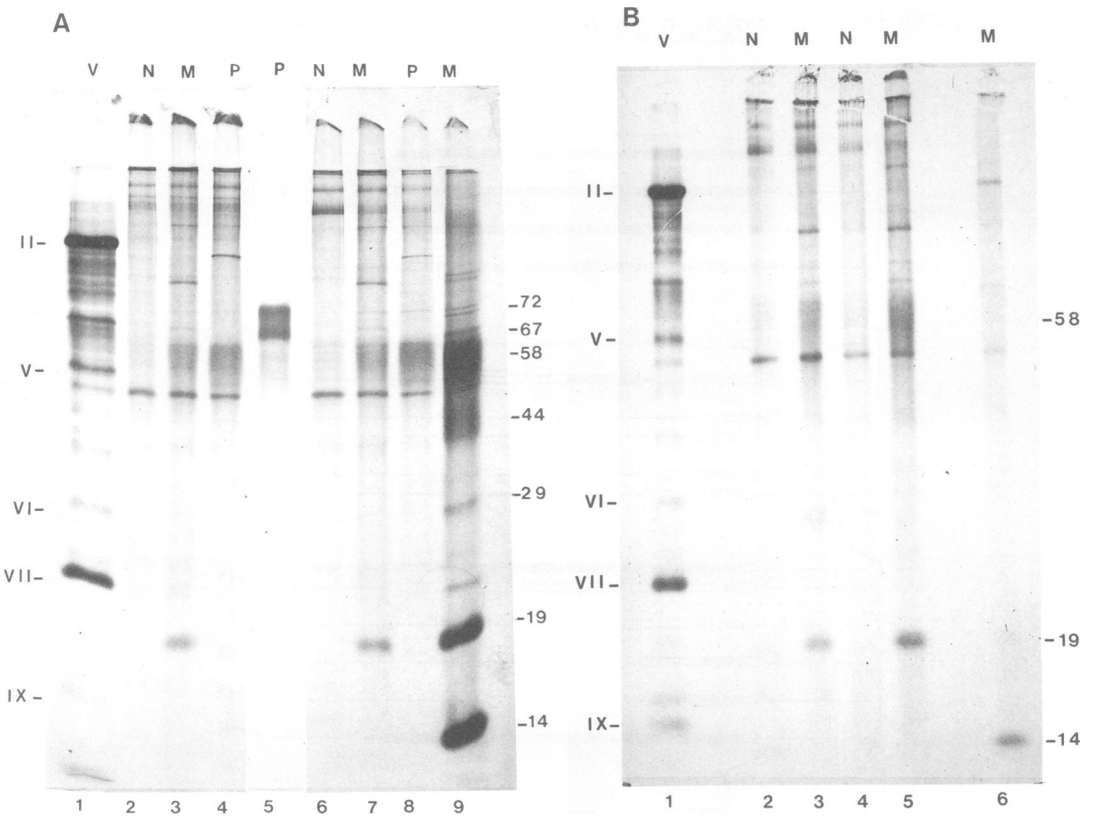

FIG. 6. Autoradiographs of SDS-polyacrylamide gels after electrophoresis of immunoprecipitated Ad5 early polypeptides. (A) [^{35}S]methionine-labeled cell extracts from transformed cell lines 1H2 (channels 2 to 4) and 637-4 (channels 6 to 8) and from virus-infected KB cells pulse-labeled from 8 to 10 h postinfection (channels 5 and 9). (B) Extracts from cell lines 5H2 (channels 2 and 3) and 637C3 (channels 4 and 5) and from virus-infected KB cells pulse-labeled from 3 to 5 h postinfection (channel 6). Abbreviations: N, Non-immune hamster serum; M, hamster Megaserum; P, rabbit P antiserum; V, [^{35}S]methionine-labeled Ad5 virus marker.

(24, 25; Rowe, personal communication), but precipitation of these proteins from transformed cells is very inefficient (Fig. 6), a finding we have consistently observed (24, 25; Rowe and Graham, unpublished data). For this reason, we cannot reach any conclusion about the expression of E1A proteins in hr-transformed cells.

DISCUSSION

Group I hr mutants of Ad5 map in the E1A region of the viral genome and are defective for transformation (10, 16, 19). These mutants are unable to transform rat embryo and rat embryo brain cells but, as shown previously (16) and in this study, they appear to induce a semiabortive or abnormal transformation of BRK cells. In this latter case, transformed cell lines could not be established from individual foci of transformed cells but could only be derived (with about a 30 to 50% success rate) by passaging entire polyclonal cultures. Once polyclonal lines had survived two or three passages, it was possible to subculture them indefinitely as well as to establish subclones. No transformed foci were observed in mock-infected cultures, and 4 of 4 attempts failed to establish continuous lines from control cultures in low-calcium medium (data not shown). Thus, although the group I mutants are defective in one or more E1A functions, they are nevertheless still capable of immortalizing cells under certain conditions.

That lines established in this way are not the result of rare cells transformed by revertants is suggested by their aberrant properties. Although after six to eight passages in culture the mutant-transformed lines achieved the same growth rates and acquired the ability to reach the same saturation densities as wild-type transformants, they differed from wild type-transformed cells in their morphology and in their inability to form colonies in soft agar. In addition, the mutant-transformed cells were either nontumorigenic or

very weakly tumorigenic in nude mice, in contrast to the majority of wild type-transformed cells. The hr mutants of group I are known to map in E1A (10) and presumably affect one or more E1A functions. Thus, these phenotypic differences between mutant- and wild type-transformed cells are presumably related to differences in the expression of E1A functions.

From studies with the hr mutants and deletion mutants of Ad5, a number of workers have reported that expression of E1A is required for transcription of other early regions of Ad5, including E1B (1, 2, 21). We have found that E1B and E2 are expressed in nonpermissive HeLa cells (24, 25; Rowe and Graham, unpublished data), albeit at reduced levels at low multiplicity of infection, and have reported that group I mutants are markedly deficient in the synthesis of a small polypeptide of molecular weight around 14,000 (previously reported as 10,500 [24, 25]). In studies with a variety of Ad5-transformed hamster cells (Rowe and Graham, unpublished data), we have been generally unable to detect E1A proteins in transformed cells, even though this region is present and transcribed and the cells can elicit an immune response against E1A proteins after injection into hamsters. Consequently, in the present studies with hr mutant-transformed rat cells, we cannot reach any conclusions on the expression of E1A proteins since they could not be detected either in mutant- or wild type-transformed cells, nor were we able to detect synthesis of the 72,000-dalton polypeptide coded by E2 in cells which contained the entire viral genome. This polypeptide has been reported to be present in Ad2 wild type-transformed hamster cells (26, 34) but not in Ad2 wild type-transformed rat cells (48). We could, however, detect the presence of the E1B polypeptides of molecular weight 58,000 and 19,000 (the latter formerly denoted 15,000 [24, 25]) at levels comparable to those seen in wild type-transformed cells (cf. Fig. 5B). Thus, E1B proteins alone are evidently not sufficient for the complete transformation of rat cells, and the explanation for the aberrant phenotype of hr-transformed cells is presumably related to the defect in E1A, suggesting that one or more E1A functions may be involved in maintenance of some of the phenotypic properties of Ad5-transformed cells. The 58,000-dalton E1B protein, on the other hand, may be involved in the initiation of cell transformation after infection with group C virions. It has been reported to be present in all of the virus-transformed cells studied to date (26, 34, 48) but is absent in cells infected by group II mutants (24, 25), which are also defective for transformation by virions (16).

One of the most interesting properties of the hr transformants was the structure of integrated viral sequences. Most lines contained a good deal more of the viral genome than was seen in our wild type-transformed lines, and many of the mutant-transformed lines appeared to contain virtually the entire viral genome colinearly integrated into host sequences. In several lines the integrated sequences extended beyond the *Hin*dIII cleavage site at 97.1 map units (i.e., within 2.9% of the right terminus) and to within less than about 1.2% of the left terminus. Whether the integrated viral sequences in these cells extend to the extreme ends of the viral genome will be clear once joint fragments are cloned and sequenced.

One rather surprising observation was that even when all DNA was extracted and analyzed at the earliest possible times after establishing mutant-transformed lines (passage four to six), the integration patterns were simple and remained stable after further subculture. Thus, although the lines were initially polyclonal, having been established from cultures usually containing 20 to 30 foci, it appears likely that by the time of the first DNA extraction only a single cell type predominated.

In all of the cell lines analyzed in the present study, the viral DNA sequences were integrated into different sites in the host DNA with the exception of three lines, 637-3, 637-4, and 809-1 (data not shown), which had been established previously (16). The pattern observed in these lines could suggest the presence of preferred sites of integration for viral DNA within the rat genome; however, the possibility of cross-contamination during the isolation of these lines cannot be ruled out.

Transformation of rodent cells by Ad2 and Ad5 generally results in lines containing only part of the viral genome (11, 37, 44). Although on occasion a larger proportion of the viral DNA is present (11, 37, 44), colinear integration of the entire genome has been observed only in exceptional situations. Dorsch-Häsler et al. (8) have recently described the integration patterns in rat cells transformed at nonpermissive or semipermissive temperature by Ad5 mutants *ts*107 and *ts*125. These mutants are DNA negative under restrictive conditions as a result of defects in the 72,000-dalton DNA binding protein (42) and transform rat cells with higher efficiency than does wild-type virus (13, 30). As we have seen with our hr mutant-transformed lines, many of the *ts*107- and *ts*125-transformed lines characterized by Dorsch-Häsler et al. appeared to contain all of the viral genome colinearly integrated (8). There are at least two other examples of adenovirus-transformed cell lines which contain the entire viral genome: these are rodent lines transformed by Ad12 (or lines established from Ad12-induced tumors) (20, 39) and a particularly nonpermissive rat line transformed by wild-type

Ad5 (H. S. Ginsberg and P. B. Fisher, personal communication).

All of these examples have features in common: they involve relatively stringent nonpermissive conditions under which functions needed for DNA replication are suppressed, and they often involve enhanced transformation efficiencies. We think a rather reasonable explanation for our findings and those of others mentioned above is that during transformation of cells by adenoviruses, usually, or perhaps always, the entire viral genome is initially integrated intact but the expression of viral functions other than those required for transformation (late genes, 72,000-dalton protein), or the replication of viral DNA, has a cytotoxic effect. In semipermissive cells infected by wild-type virus, presumably only those cells which retain a defective genome as a result of deletion or rearrangement of viral DNA sequences are able to survive. When a mutation prevents the expression of putative cytotoxic functions (e.g., hr group I; *ts*107, *ts*125) or when the cells are totally nonpermissive to the virus, then survival of transformed cells containing the entire intact viral genome becomes possible.

Consistent with this hypothesis is the extensive cytopathic effect seen in BRK cultures infected with wild-type Ad5 but not with the group I hr mutants. In addition, we have seen that the efficiency of transformation of BRK cells by wild-type Ad5 drops rapidly at multiplicities of infection higher than 1 (presumably as a consequence of cell killing), whereas the efficiency of transformation by hr mutants continues to increase.

If colinear integration of the entire viral genome into host DNA is a common mechanism by which integration initially occurs during transformation by virions, then mutant-transformed cells in which subsequent rearrangements or deletions have not occurred may be useful tools for studying the integration process.

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