Transformation of Rat Cells by the Hybrid Virus Ad2^{2+} HEY

BY DENISE A. GALLOWAY, EUGENE LUKANIDIN*
WILLIAM C. TOPP AND JOE SAMBROOK
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, U.S.A.

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SUMMARY
A set of four isogenic rat cell lines transformed by Ad2^{2+} HEY have been studied. While all of the cell lines synthesize SV40 T antigen, only one expresses adenovirus 2 T antigen: none expresses SV40 V antigen or adenovirus 2 fibre antigen. Three cell lines contain 1 to 2 virus equivalents of SV40 and adenoviral sequences per diploid quantity of rat cell DNA and the fourth line contains five copies of SV40 and 20 copies of the adenovirus genome. At least three of the cell lines contain DNA sequences from the helper adenovirus 2 in addition to sequences from the Ad2^{2+} HEY genome. The patterns of integrated virus sequences are complex suggesting multiple insertions of both adenovirus and SV40 DNA sequences. SV40 can be rescued from three cell lines by fusion with permissive cells.

INTRODUCTION
Attempts to propagate adenoviruses in cultures prepared from rhesus monkey tissue led to the generation and subsequent discovery of hybrid viruses (Huebner et al. 1964; Rapp et al. 1964; Rowe & Baum, 1964; Easton & Hiatt, 1965; Lewis et al. 1966a, b; Morris et al. 1966) whose recombinant genomes consist of SV40 DNA integrated at specific sites within adenovirus 2 DNA. The hybrid viruses fall into two classes: the non-defective viruses which are capable of independent replication and contain different quantities of the early region of the SV40 genome inserted into adenovirus 2 DNA between map positions 0.80 and 0.86 (Lewis et al. 1973, 1974a, b). The second class of hybrids is defective and replicates only in the presence of a helper virus such as adenovirus 2. One of these, defective virus Ad2^{2+} HEY, is a heterogeneous population containing wild-type adenovirus 2 and three hybrid species which have 0.4, 1.4 and 2.3 copies of the SV40 genome integrated into adenovirus 2 DNA between positions 30 and 75 (Kelly et al. 1974). Additionally, two new unrelated hybrid species have been isolated from an Ad2^{2+} HEY pool, Ad2^{D1} and Ad2^{D2}, which contain fractional lengths of the SV40 genome integrated into other sites of adenovirus DNA (Hassell et al. 1978).

Although rodent cells transformed by SV40 almost invariably contain the entire virus genome, only a subset of SV40 genes is required for transformation. The product(s) of virus genes located between positions 67 and 17 on the SV40 map are necessary for transformation and restriction fragments of DNA containing this region have been shown to be sufficient for transforming rodent cells (Graham et al. 1974; W. Topp, unpublished data). By contrast, rodent cells transformed by adenovirus 2 do not contain a complete set of virus genes (Sambrook et al. 1974). A segment that begins at, or close to, the left-hand end

* Present address: Fred Hutchinson Cancer Research Centre, Seattle, Wa., U.S.A.
† Present address: Institute for Molecular Biology, Moscow, U.S.S.R.
of the virus DNA and extends to a point lying about 7.5\% along the adenovirus 2 genome appears to be sufficient for transformation (van der Eb et al. 1977). Other cell lines contain more extensive although never complete collections of virus sequences. We thought it would be interesting to study the interaction between rat cells and hybrid adeno-SV40 viruses which have the potential to transform by expressing either adenovirus or SV40 sequences. The Ad22+ HEY genome has been mapped by heteroduplex analysis and the results showed that adenovirus 2 sequences from 0 to 14\% and SV40 virus DNA sequences from map positions 67 to 17 are present in the hybrid genome (Kelly et al. 1974).

Rodent cells have been transformed previously by hybrid viruses and the pattern of expression of virus antigens varied with different viruses. Hamster kidney cells were transformed with all five of the non-defective adeno 2–SV40 hybrid viruses (Ad2+ND1–Ad2+ ND5; Lewis et al. 1974a, b). All of the lines studied had adenovirus 2 T antigen and virus-specific RNA, but expression of the SV40 genome was variable, with only one line containing detectable amounts of SV40 antigens. On the other hand, hamster embryo fibroblasts transformed by the PARA (defective SV40)–adenovirus 7 hybrid population (Huebner et al. 1964; Black & Todaro, 1967) produced SV40 T antigen and seldom showed any adenovirus 7 T antigen (Rapp et al. 1964; Diamond, 1967).

We have transformed rat embryo cells with DNA from an Ad22+ HEY virus population. We chose to transform with DNA rather than intact virus because the purity of the DNA used could be determined easily. The studies presented here have investigated the phenotypic characteristics of the transformed cells, the virus antigens synthesized by the transformants, rescue of SV40 genomes, the content of virus DNA and the arrangement of integrated virus DNA sequences.

METHODS

Viruses. Ad22+ HEY originally obtained from A. M. Lewis in 1973 was propagated on monolayers of CV1 cells infected at low multiplicity (1 p.f.u./cell). Before infection, the virus stocks were incubated at 37 °C for 20 min with an equal volume of SV40 neutralizing serum (Flow) diluted 1:10. Virions were purified by equilibrium centrifugation in CsCl and iothalamate gradients (Serwer, 1975). Adenovirus type 2 was propagated in suspension cultures of HeLa cells. SV40 was propagated on monolayers of CV1. The DNAs of Ad22+ HEY and adenovirus 2 were isolated from purified virus particles (Pettersson & Sambrook, 1973). The purity of Ad22+ HEY DNA was analysed by restriction digests and by hybridization and showed that 1\% of the DNA was from adenovirus 2 and contained less than \(10^{-6} \mu g\) of SV40 DNA per \(\mu g\) of Ad22+ HEY DNA. SV40 DNA was isolated by the Hirt extraction procedure (Hirt, 1967) and purified by equilibrium gradient centrifugation in CsCl containing ethidium bromide.

Isolation and growth of transformed cell lines. Primary cells prepared from fourteen day Fisher rat embryos were infected with Ad22+ HEY DNA (10 \(\mu g/60 \) mm plate) using the calcium phosphate precipitation technique of Graham & van der Eb (1973). After 3 to 4 weeks, feeding twice a week but without subculture, transformants appeared as dense foci on the cell monolayer. These were teased from the plate with a Pasteur pipette, trypsinized and re-plated. Single cell lines were obtained by cloning through microwells (Linbro). Some of the dishes were maintained in medium which was low in calcium and kept for 6 to 8 weeks with no obvious affect.

All cells were cultured in an atmosphere of 10\% CO₂ using Dulbecco’s modification of Eagle’s medium (DME; Biorad) with 10\% foetal calf serum (Irvine) on plastic tissue culture plates (Lux and Falcon).
Transformation by Ad2<sup>2+</sup> HEY

**Growth assays.** To obtain growth curves, 12 replica 35 mm plates were seeded on day 0 with 10<sup>5</sup> cells/plate. On day 1 the medium was removed from six plates and replaced with DME containing 1% FCS. Cells were counted every other day starting on day 2 and were fed every 3 days.

To determine saturation densities, 35 mm plates were seeded with 10<sup>6</sup> cells/plate, fed twice weekly and counted on day 10.

Growth in semi-solid medium was assayed by plating 10<sup>5</sup> cells on plates coated with 0.9% methylcellulose. Cells were fed weekly and colonies were counted after 3 weeks.

**Preparation of DNA and RNA from transformed cells.** Cells grown to confluence in DME containing 5% FCS were removed from the dish by scraping, concentrated by centrifugation and washed twice with ice-cold phosphate buffered saline (PBS). DNA was extracted as described (Botchan & McKenna, 1973). To prepare 32P-labelled transformed cell RNA, the cells were grown in DME lacking phosphate for 4 h and then incubated for 16 h with 1 mCi of 32P-orthophosphate/plate (New England Nuclear, sp. act. 250 Ci/mmol). The cells were removed from the dish by scraping and washed three times with ice-cold PBS. Cytoplasmic RNA was extracted by the method of Craig & Raskas (1974).

**Immunofluorescence tests.** Cell lines were grown to subconfluency on glass coverslips, washed in PBS, fixed in 3.5% formaldehyde and dehydrated for 10 min in cold acetone (−20 °C). For visualization of SV40 T antigen the cells were incubated with hamster anti-SV40 T antigen (Flow) and fluorescein isothiocyanate conjugated (FITC) goat anti-hamster IgG (Antibodies, Inc.). For detection of SV40 V antigen, cells were incubated with rabbit neutralizing antibody (Flow) which had been pre-adsorbed with partially purified SV40 T antigen and a pre-immune cell extract, and FITC labelled goat anti-rabbit IgG (Meloy). For visualization of adenovirus 2 T antigen the cells were treated with hyperimmune sera from rats injected with the adenovirus 2 transformed cell line Ad2/F17 (a gift of Jim McDougall) and FITC labelled goat and anti-rat IgG (Meloy). For detection of adenovirus 2 fibre antigen the cells were treated with sera from rabbits injected with purified adenovirus 2 fibre (a gift of Carl Anderson) and FITC labelled goat anti-rabbit IgG (Meloy).

**Immunoprecipitation of virus antigens.** Cells were grown in DME lacking methionine for 3 h and then incubated at 37 °C for 1 h with 100 μCi of 35S-methionine/plate (New England Nuclear, sp. act. 600 Ci/mmol). The medium was removed and the cells were washed three times with ice-cold PBS containing phenyl methyl sulphonyl fluoride (PMSF). The cytoplasm was prepared as described by Prives et al. (1977). Immunoprecipitation of the SV40 T antigens was carried out as described by Tjian (1978) using sera from tumour-bearing hamsters induced by SV40 and rabbit anti-hamster IgG (Cappal). The precipitates were fractionated by electrophoresis through 7 to 20% gradient polyacrylamide gels (Tjian, 1978), dried and exposed for autoradiography.

**Virus rescue.** Transformed cells (10<sup>5</sup>) and CVI cells (10<sup>6</sup>) were grown together on a Petri dish for 1 day and then fused with polyethylene glycol (PEG; Pontecorvo, 1975) including dimethyl sulfoxide (DMSO; Norwood et al. 1976). The cells were washed three times with medium containing DMSO and once with medium containing 10% FCS. After 48 h the fusion lysate was titred on monolayers of CV1 cells.

**Restriction enzymes.** All of the restriction enzymes used were prepared by Ronni Greene. The endonucleases EcoRI, BamHI, Hha and HaeII and III were prepared according to published procedures (Yoshimori, 1971; Middleton et al. 1972; Lebowitz et al. 1974; Wilson & Young, 1975; Roberts et al. 1976). Endonuclease BglII and II, SmaI and Kpn were isolated by the unpublished procedures of G. Wilson & F. Young, R. Green and C. Mulder, and D. I. Smith. DNA was digested at 37 °C in a total volume of 0.05 to 10 ml.
for periods of 2 to 10 h depending on the nuclease used. The reaction mixtures contained the following: for endonuclease EcoRI—0.05 M-tris-HCl (pH 7.8), 0.01 M-MgCl₂; for endonuclease SmaI—0.05 M-tris-HCl, pH 9.0, 0.015 M-KCl, 0.005 M-MgCl₂; and for all other endonucleases—0.01 M-tris-HCl (pH 7.7), 0.01 M-MgCl₂ and 0.001 M-dithiothreitol.

**DNA reassociation.** The preparation of DNA from transformed cells, purification of fragments of virus DNA for use as hybridization probes, hybridization conditions, and detection of the hybrids by hydroxylapatite chromatography was carried out as previously described (Sharp et al. 1974b). Instead of using virus DNA which was labelled in vivo, fragments of unlabelled virus DNA were prepared and then made radioactive in vitro (see below).

**Gel electrophoresis, blotting, nick translation and hybridization.** Gel electrophoresis was carried out in vertical slab gels cast with 1.0 % agarose as described by Sugden et al. (1975). The gel was stained with ethidium bromide (0.5 μg/ml in electrophoresis buffer) and photographed through a Kodak 22A filter on to Polaroid film using ultraviolet illumination (Sharp et al. 1973). The DNA was denatured in situ and transferred to nitrocellulose sheets following the procedure of Southern (1975) as previously described (Botchan et al. 1976). Introduction of ^32P-nucleotides into virus DNA to use as hybridization probes was carried out as described (Maniatis et al. 1976). DNA polymerase I was purchased from Boehringer-Manheim Biochemicals.

The purification of the probe and tests of its integrity were carried out as described (Botchan et al. 1976). Specific activities of 5 x 10⁷ to 1 x 10⁸ were routinely obtained. The hybridization procedure, pre-treatment of the nitrocellulose filters and their subsequent washing were performed as described (Botchan et al. 1976). ^32P-labelled cytoplasmic RNA which had not been poly-A selected was hybridized to pretreated nitrocellulose filters in the same manner in which hybridizations with DNA probes were performed. At the end of 18 h the filters were treated with RNase A (50 μg/ml, Worthington) at 37 °C for 2 h followed by washing in 2 × SSC, 0.5 % SDS.

### RESULTS

**Restriction map of Ad2⁺ HEY**

The structure of the hybrid genomes in Ad2⁺ HEY was determined by heteroduplex mapping by Kelly et al. (1974). Restriction maps were constructed to pinpoint further the virus sequences present in the hybrid genome. When DNA from an Ad2⁺ HEY pool was digested with various restriction endonucleases, fractionated by electrophoresis through agarose gels and stained with ethidium bromide, the pattern obtained was that of adenovirus 2 DNA. To determine the positions of the SV40 sequences, the DNA in the gel was denatured in situ, transferred to nitrocellulose filters, hybridized to ^32P-labelled SV40 DNA and exposed for autoradiography. The pattern obtained is shown in Fig. 1. Knowing the positions of cleavage by the restriction endonucleases on the DNAs of SV40 and adenovirus 2 combined with the heteroduplex map of Ad2⁺ HEY obtained by Kelly et al. (1974), a restriction map was deduced for the hybrid virus. For example, SV40 DNA is cleaved once by BamHI at position 14 and adenovirus 2 DNA is cleaved three times at positions 29.1, 40.9 and 59.0. When Ad2⁺ HEY was cleaved with BamHI, three fragments which contain SV40 DNA were generated: one which co-migrated with unit length SV40 DNA, one with a mol. wt. which was 95 % of unit length SV40 DNA and a third fragment with a mol. wt. of 7.5 x 10⁶ (see Fig. 1). The SV40 linear size fragment corresponds to the internal SV40 genome present in a molecule of Ad2⁺ HEY III. The smaller fragment corresponds
Transformation by $Ad^2+\ HEY$

Fig. 1. Detection of fragments of DNA that contain SV40 sequences after hydrolysis of $Ad^2+\ HEY$ DNA with restriction endonucleases. One $\mu$g of $Ad^2+\ HEY$ DNA was used in each digestion in a total volume of 0.1 ml. The products of the digestion were fractionated by electrophoresis in vertical slab gels through 1% agarose at 15 h at a potential of 1.5 V/cm, transferred to a nitrocellulose filter, hybridized to $^{32}$P-labelled SV40 DNA and exposed for autoradiography.

to the DNA extending from position 14 on the SV40 genome leftwards to position 29.1 on the adenovirus genome. The third fragment corresponds to a piece of DNA which extends from the right-most SV40 BamHI cleavage site to the right end of the adenovirus 2 genome. Using a variety of restriction enzymes a more complete map was determined as shown in Fig. 2. The approximate junction points are positions 30 and 73.5 on the adenovirus genome and positions 22 and 65 on the SV40 genome.

Properties of HEYR cell lines

DNA virus transformants are commonly defined by an alteration in growth properties. Cultures of cells transformed by SV40 are able (1) to grow to high cell densities, (2) to proliferate in medium low in serum (1-0% FCS) and (3) to form colonies in Methocel with efficiencies at least 1% of that obtained on plastic surfaces. Adenovirus 2 transformants are often composed of a heterogeneous population of epithelioid cells which grow to high cell densities, proliferate in low serum, have a high plating efficiency in agar, but have generation times slightly longer than the control cells (Gallimore, 1974). Transformants obtained with the left-end 7.5% DNA fragments show very low (0-03 to 0.85%) cloning efficiency in agarose (van der Eb et al. 1977). When the HEYR cell lines were tested by
these criteria the results shown in Table 1 were obtained. All four HEYR cell lines are able to grow to high saturation densities, grow well in low serum and clone in Methocel.

The types of virus specific antigens present in the HEYR cell lines were determined by immunofluorescence microscopy using specific antisera. The classic nuclear staining pattern of SV40 T antigen was seen in all four of the HEYR cell lines whereas no SV40 V antigen or adenovirus 2 fibre antigen could be detected in any of the cell lines. Only one line, HEYR 4 gave a specific staining pattern with antisera against adenovirus 2 T antigen as shown in Fig. 3. The pattern of virus antigen expression suggests that the HEYR cell lines were transformed by the SV40 moiety of the hybrid virus.

To determine whether the virus T antigen in the HEYR cell lines is similar to that produced by wild-type SV40 in a lytic infection, the mol. wt. of proteins specifically precipitated by SV40 antisera was determined by electrophoresis on SDS-polyacrylamide gels as shown in Fig. 4. A small T antigen of 17K (Prives et al. 1977) was precipitated from all of the transformed cell lines. The large T antigen present in cell lines HEYR 1, HEYR 3 and HEYR 4 is indistinguishable in size from that produced in SV40-infected monkey cells (96 to 97 K; Tegtmeier et al. 1975); however, when large T antigen was immunoprecipitated from HEYR 2, a protein with an apparent mol. wt. of 93 to 94 K was seen (Fig. 4b).
Fig. 3. Immunofluorescence of adenovirus 2 T antigen. Specific fluorescence was seen in the cell lines (a) Ad2/F17 and (c) HEYR 4 using Ad2 T-ag antiserum prepared against Ad2/F17 and FITC labelled rabbit anti rat IgG. No fluorescence was seen in the cell lines HEYR 1 (b), HEYR 2 and HEYR 3 (not shown).
Fig. 4. Immunoprecipitation of SV40 T antigen from HEYR cell lines. (a) For each cell line, a 90 mm plate was grown to confluency in DME containing 5% PBS, starved for 3 h in medium lacking methionine, and labelled for 1 h at 37 °C with 100 μCi of 35S-methionine (NEN). The cytoplasm was prepared as described by Prives et al. (1977) and incubated with 10 μl of SV40 anti-T antiserum (Tjian, 1978) for 90 min at 4 °C followed by an overnight incubation at 4 °C with 150 μl of rabbit anti-hamster IgG (Cappel). The immunoprecipitates were fractionated by electrophoresis through 7 to 20% gradient polyacrylamide gels (Tjian, 1978). The slot marked Ad2 proteins is a 35S-labelled extract of Ad2 infected HeLa cells used as a marker for mol. wt. determinations. The slot marked SV40 inf. cells is a 35S-labelled extract wild type SV40 infected CV1 cell (a gift of Merilyn Sleigh). (b) A 90 mm plate of cells of each transformed cell line was labelled with 50 μCi of 35S-methionine overnight and nuclei were prepared (Tjian, 1978). SV40 T antigen was precipitated and fractionated on 7 to 15% gradient polyacrylamide gels as described above.
Transformation by Ad2^z+ HEY

Rescue of SV40 from the HEYR cell lines

In transformed cells which contain at least one complete virus genome, SV40 can be recovered by fusion of the transformed cell with a permissive monkey cell (Watkins & Dulbecco, 1967). Fusion between the HEYR cells and CV1 cells was carried out as described in Methods and the yield of virus was determined by titration of the fusion lysate on monolayers of CV1 cells. The cell lines HEYR 1, HEYR 3 and HEYR 4 yielded 10^4 to 10^5 p.f.u./ml. To prove that the virus obtained by rescue was SV40 and to determine whether the virus was perfect, the DNAs of 30 plaque purified viruses obtained by fusion were analysed following digestion by the endonuclease HaeIII (data not shown). The pattern obtained for all of the virus DNAs was identical to that of wild-type SV40. Had imprecise excision occurred, adenovirus DNA sequences might be present in the virus genome obtained by rescue. To test this, 10 µg of each rescued virus DNA was digested with restriction endonucleases, fractionated by electrophoresis, transferred to nitrocellulose filters, and hybridized to ^32P-labelled adenovirus 2 DNA (5 × 10^7 ct/min/µg). No adenovirus DNA sequences were detected in the rescued genomes under conditions which were able to detect 10^-6 µg of adenoviral DNA in a reconstruction (data not shown).

Rescue of SV40 from HEYR 2 was unsuccessful in several attempts. Fusion of HEYR 2 with CV1 cells infected with a SV40 ts A mutant also failed to yield virus. There are several possible explanations for the failure of HEYR 2 to yield virus following fusion with a permissive cell: an incomplete SV40 genome is present in this cell line; there is a defect in the late region of the genome which prevents synthesis of virion proteins or packaging; or HEYR 2 is incapable of excision of virus DNA and/or replication. The first possibility does not appear to be correct because the integrated virus DNA sequences contain at least one intact genome (see later section). If there is a defect in the late region which prevents virion formation, it is possible that virus DNA would be excised and replicated following fusion. Virus DNA has been detected following fusion of a SV40-transformed cell which failed to yield virus after fusion (M. Botchan, personal communication). To determine if virus DNA is present in the heterokaryons, low mol. wt. DNA was obtained by the Hirt (1967) extraction procedure at various times after fusion, fractionated by electrophoresis through agarose gels, transferred to nitrocellulose sheets, hybridized to ^32P-labelled SV40 DNA and the position of the virus DNA determined by autoradiography. By 60 h after fusion no free virus DNA was seen in the lysate of HEYR 2 whereas in other cell lines, virus DNA was detected 8 h after fusion (data not shown). These results suggest that either the integrated virus DNA was not excised from the rat cell genome or it was unable to replicate in the HEYR 2 heterokaryon.

Sequences of virus DNA present in transformed cells

To assay DNA extracted from transformed cells for sequences homologous to each of the BamI fragments of adenovirus 2 DNA and the fragments of SV40 DNA generated by combined cleavage with BamHI, BgII and HaeII, the rate of reannealing of the ^32P-labelled fragments was measured in the presence of unlabelled DNA extracted from the HEYR cells. Calf thymus DNA used in these experiments as a control has been shown not to accelerate the rate of reannealing of adenovirus 2 DNA (Pettersson & Sambrook, 1973). The results of one complete experiment are shown in Fig. 5 in which the kinetics of renaturation of each of the specific fragments of adenovirus 2 DNA and SV40 DNA were measured in the presence of (a) DNA extracted from the HEYR cells; (b) calf thymus DNA; and (c) calf thymus DNA to which the equivalent of 2 copies per cell of adenovirus 2 DNA, or 1 or
Fig. 5. Kinetics of reassociation of $^{32}$P-labelled fragments of adenovirus 2 DNA produced by cleavage with BamHI and with $^{32}$P-labelled fragments of SV40 DNA produced by cleavage with BamHI, BglII and HaeIII in the presence of calf thymus (C.T.) DNA and DNA extracted from the HEYR cell lines. The conditions of the hybridization reactions and the calculation of the renaturation times are as described by Sharp et al. (1974a, b).
Transformation by \( \text{Ad}^{2+} \) HEY

Table 2. *Virus DNA sequences in rat cells transformed by \( \text{Ad}^{2+} \) HEY*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Adenovirus BamHI fragments</th>
<th>SV40 BamHI-BgII-HaeII fragments</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td>HEYR 1</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>HEYR 2</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>HEYR 3</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>HEYR 4</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

The arrangement of integrated virus DNA sequences

The location of virus DNA sequences among the products of digestion of transformed rat cell DNA by restriction endonucleases was determined by hybridization. High mol. wt. DNA purified from four lines of transformed rat cells was hydrolysed by various enzymes. The mixture was divided into two sets and the fragments were separated by electrophoresis through agarose gels, transferred directly to nitrocellulose sheets; one set was hybridized to \(^{32}P\)-labelled SV40 DNA and the other to \(^{32}P\)-labelled adenovirus 2 DNA. The results obtained with two enzymes, *SmaI* and *EcoRI*, are given in Fig. 6 and 7.

The overall impression is one of complexity. It is not possible to draw detailed maps of the integrated virus DNA sequences; however, the following conclusions can be reached:

(i) DNA sequences from the hybrid virus, \( \text{Ad}^{2+} \) HEY are present in the DNA of the transformants. All four of the HEYR cell lines show radioactive bands which co-migrate with authentic fragments of \( \text{Ad}^{2+} \) HEY DNA. The endonuclease *EcoRI* cleaves \( \text{Ad}^{2+} \) HEY III DNA five times, twice within the SV40 sequences yielding an SV40 unit length fragment and two fragments containing the junction between adenovirus 2 and SV40 sequences, and three times within the right-hand adenovirus 2 sequences. The fragment containing the right-hand junction of the adeno-SV40 sequences migrates faster than unit length SV40 DNA and can be detected in a reconstruction using \( 10^{-5} \)\( \mu \)g of \( \text{Ad}^{2+} \) HEY DNA and in the DNAs of HEYR 1, HEYR 3, and HEYR 4 (see Fig. 7). Similarly, among the products of cleavage of HEYR 2 DNA with the endonuclease BamHI, a fragment which...
Fig. 6. Detection of fragments of DNA that contain SV40 sequences after hydrolysis of transformed rat cell DNA with endonuclease Smal. High mol. wt. cell DNA was extracted from the transformed cells and 5 μg of DNA were digested with Smal for 4 h at 37 °C in 0.10 ml. The products of digestion were fractionated, transferred to nitrocellulose filters and hybridized to 32P-labelled SV40 DNA as in Methods. The slots labelled Rat, Rat + HEY and Rat + SV40 each contained 5 μg of DNA extracted from rat primary cells mixed with 10⁻² μg of Ad2 HEY DNA and SV40 DNA, respectively.
Transformation by Ad\textsuperscript{2+} HEY

Fig. 7. Detection of fragments of DNA that contain SV\textsubscript{40} and adenovirus 2 sequences after hydrolysis of transformed rat cell DNA with endonuclease EcoRI. High mol. wt. cell DNA was extracted from the transformed cells and 5 μg of DNA were digested with EcoRI for 2 h at 37 °C in 0-10 ml of a reaction mixture containing 0.05 M-tris-HCl (pH 7.8), 0.01 M-MgCl\textsubscript{2}. The products of digestion were fractionated by electrophoresis, transferred to nitrocellulose and hybridized as in Fig. 3. The filter was cut into two parts and one half was hybridized to \textsuperscript{32}P-labelled SV\textsubscript{40} DNA and the other half was hybridized to \textsuperscript{32}P-labelled adenovirus 2 DNA. The slots labelled Ad\textsuperscript{2+} HEY reconstruction, SV\textsubscript{40} reconstruction and Ad2 reconstruction each contained 5 μg of DNA extracted from Fisher rat primary embryo cells mixed with 10\textsuperscript{-5} μg Ad\textsuperscript{2+} HEY DNA, SV\textsubscript{40} DNA and adenovirus 2 DNA, respectively.

co-migrates with an authentic fragment of Ad\textsuperscript{2+} HEY DNA can be detected (data not shown). These radioactive bands which represent junction fragments between adenovirus and SV\textsubscript{40} DNA sequences are seen when hybridized to either \textsuperscript{32}P-labelled Ad2 DNA or \textsuperscript{32}P-labelled SV\textsubscript{40} DNA (Fig. 7).

(2) The patterns of integration in some of the cell lines (HEYR 2 and 3) can be explained by a single insertion of Ad\textsuperscript{2+} HEY, whereas in other cell lines the patterns suggest multiple insertion sites. Fig. 6 shows the distribution of sequences which hybridize to SV\textsubscript{40} following
hydrolysis by Smal, an endonuclease which, because it does not have a recognition site within SV40 DNA, yields a total number of radioactive bands at least equal to the number of independent insertions of SV40 sequences. Whereas the DNAs from HEYR 2 and 3 each show only one radioactive band in a position of high mol. wt. DNA, the patterns seen for HEYR 1 and 4 are complex; each line contains several insertions. Not all of the radioactive bands can be attributed to Ad2\textsuperscript{+} HEY DNA sequences.

(3) All four of the transformed cell lines appear to contain virus sequences from the Ad2\textsuperscript{+} HEY III genome or possibly from some other structure from which unit length SV40 can be cleaved. Digestion of Ad2\textsuperscript{+} HEY III DNA, which contains 2·4 copies of SV40 in tandem with an endonuclease that cleaves SV40 DNA at a single site, produces a unit length SV40 fragment. Such a fragment was seen in a reconstruction on digestion with EcoRI (Fig. 7), BamHI, BglII and Kpn (data not shown) following hybridization with \textsuperscript{32}P-labelled SV40 DNA. A radioactive band which co-migrates with unit length SV40 was detected in the DNA of all four cell lines following digestion with the same endonucleases. No free SV40 DNA was detected when DNA from the transformed cells either was not digested (data not shown) or was digested with an enzyme which does not cleave SV40 Smal (Fig. 6). These data suggest that unit length SV40 in DNA from the transformed cells has been cleaved from a larger piece of DNA, possibly from an Ad2\textsuperscript{+} HEY III genome.

(4) There does not appear to be a unique site of integration into the rat cell genome. Radioactive bands in the position of high mol. wt. DNA which do not co-migrate with authentic virus fragments can be detected. Presumably these bands represent the junction between virus and host cell sequences. The positions of these bands vary among the cell lines suggesting that there is no unique site of integration into the rat cell genome.

Detection of virus specific RNA

To determine which regions of the virus genome are transcribed into messenger RNA, \textsuperscript{32}P-labelled RNA extracted from the cytoplasm of the transformed cells was hybridized to nitrocellulose sheets containing restriction fragments of adenovirus 2 or SV40 DNA. The pattern obtained by hybridization to the SV40 sequences was identical in all four cell lines and is shown in Fig. 8 (e). There is hybridization only to fragments within the early region falling between SV40 map positions 67 to 17. The pattern of hybridization to adenovirus 2 DNA fragments varied among the four cell lines. In HEYR 1 and 2 (Fig. 8a and b) no adenovirus 2 specific RNA could be detected. In the cytoplasm of HEYR 3 (Fig. 8c), RNA which hybridized to the DNA of EcoRI fragments D and E, HindIII fragments E and H, BglIII fragments F and I, and Smal fragment C was detected corresponding to a region between map positions 75·9 and 89·5 on the adenovirus genome. Other radioactive bands seen in this panel resulted from hybridization to partial products of restriction digests. A transcript from that same region of the genome has been found in the cytoplasm early during lytic infection (Sharp et al. 1974a) and may be comparable to the adenovirus specific RNA found in HEYR 3. In the cytoplasm of HEYR 4 (Fig. 8d), RNA which hybridized to virtually the entire adenovirus genome was detected. This finding is consistent with the observation that HEYR 4 is the only cell line in which adenovirus 2 T antigen can be shown by immunofluorescent staining.
Fig. 8. Analysis of virus specific cytoplasmic RNA from HEYR cell lines. Panels (a to d) contain restriction fragments of adenovirus 2 DNA, to which 32P labelled RNA extracted from the cytoplasm of the cell lines HEYR 1 (a), HEYR 2 (b), HEYR 3 (c), and HEYR 4 (d) has been hybridized. Panel (e) contains SV40 DNA fragments to which 32P-RNA from any of the four cell lines was hybridized. Each channel contains 1.0 μg of virus DNA digested with the specified restriction endonuclease. Approx. 10^7 ct/min of 32P-cytoplasmic RNA was hybridized to each filter for 18 h at 68 °C.
DISCUSSION

This paper presents data which demonstrate that Ad2 HEY is able to cause the *in vitro* transformation of rat cells. In a population which contains several types of virus genome—adenovirus 2, Ad2 HEY types I, II and III (Kelly *et al.* 1974), possibly SV40 (Lewis & Rowe, 1970), and Ad2+D1 and Ad2+D2 (Hassell *et al.* 1978)—it was necessary to determine which genome was responsible for the observed transformation. In addition, the hybrid genomes contain the genetic information for transformation by both SV40 and adenovirus 2. In the cell lines, HEYR 1 and HEYR 3 it is clear that transformation was directly related to the SV40 portion of the hybrid genome. The SV40 T antigen which is made by HEYR 2 is probably responsible for maintaining a transformed cell phenotype; however, its altered size and the inability to rescue virus from HEYR 2 raise questions. It is not unusual, however, for cells which are commonly believed to be transformed by SV40 to produce T antigens of various sizes. Both SV40 and adenovirus T antigens are elaborated in HEYR 4 cells. Therefore, it is impossible to determine which virus sequence was responsible for transformation.

It is clear from the restriction cleavage patterns of the transformed cell DNA that the major component in the transformation was Ad2 HEY III although adenovirus 2 appears to have been present as well. Hassell *et al.* (1978) have shown that Ad2+D1 and Ad2+D2 are very minor components of the Ad2 HEY population; and although sequences from these hybrid genomes have not been demonstrated in the DNA of the transformed cells and do not seem a likely possibility, the presence of Ad2+D1 and Ad2+D2 cannot be ruled out entirely. The evidence presented indicates that Ad2 HEY sequences are integrated into the DNA of the HEYR cell lines and cause these cells to express virus antigens and have the phenotype of transformed cells.

The virus antigens which were detected are characteristic of transformed cells in that early antigens were found, e.g., both small and large SV40 T antigens and adenovirus 2 T antigen, but no late antigens, e.g., SV40 V antigen or adenovirus 2 fibre antigen. The data obtained by renaturation kinetics (see Fig. 5) indicate that the left-hand sequences of the adenovirus 2 genome which code for T antigen are present in all four lines examined. Thus, the presence in transformed cells of virus DNA sequences which code for early gene products does not assure transcription or translation. Similarly, only HEYR 3 makes a specific RNA from the right-hand region of the adenovirus 2 genome although these sequences are present in all of the lines. It is also notable that synthesis of SV40 T antigen did not prohibit cells from expressing adenovirus 2 T antigen as well. The interaction between the virus and host cell genomes in transformed cells must be complex with many factors regulating the expression of virus genes.

The HEYR cell lines offer the interesting possibility of studying the regulation of virus gene expression. One method by which previously inactive genes may be turned on occurs during the formation of tumours. The HEYR cells were inoculated into nude mice and all of the lines produced tumours. Preliminary evidence indicates that the tumour cells are synthesizing adenovirus 2 T antigen in addition to SV40 T antigen. Further studies are in progress to characterize the factors which modulate virus gene expression in transformed and tumour cells.
Transformation by Ad22+ HEY

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