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Transforming Genes of Canine Adenovirus Type 2

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SUMMARY
Genes of canine adenovirus type 2 (CAd2) involved in transformation were localized and their functions investigated. Cells transformed by CAd2 whole DNA or the restriction enzyme fragment (leftmost 47%) totally lacked contact inhibition and were serum-independent, anchorage-independent and tumorigenic in newborn rats. All the cells transformed by the XbaI D (0 to 15.2%) and the EcoRI C (0 to 11.3%) fragments were morphologically transformed, but were serum-dependent, anchorage-dependent and not tumorigenic in newborn rats after 150 and 190 days observation, respectively, when 5 × 10⁶ cells per rat were injected. No transforming activities were detected by DNA fragments smaller than EcoRI C. By Northern blot hybridization, it was shown that a 1 kb mRNA was encoded in the 0 to 45% region of the genome, and 1.1 kb and 2 kb RNAs in the 4.5 to 11% region. It was therefore suggested that these gene products are required for morphological transformation, and other gene(s) or element(s), which have not been identified, may be involved in serum independence, anchorage independence and tumorigenicity of the transformed cells.

INTRODUCTION

The early region 1 (E1) of human adenoviruses (Ads) has been well investigated because it shows interesting features in at least the following aspects. First, it participates in transformation of rodent cells, which has been proposed to be a multi-step process (Land et al., 1983; Ruley, 1983). It has been shown that the genes encoded in the left-hand region of the viral genome contribute to the transformation of rodent cells. The E1A region has an immortalizing activity in primary cultured cells (Hurwitz & Chinnadurai, 1985a; Montell et al., 1984; Moran et al., 1986; van den Elsen et al., 1982, 1983b; Zerler et al., 1986) and contributes to some extent to the morphological change (Roberts et al., 1985; van den Elsen et al., 1983b), and at least part of the E1B region is required in addition for complete transformation (Bernards et al., 1982; Hurwitz & Chinnadurai, 1985b; Montell et al., 1984; Ruley, 1983; van den Elsen et al., 1982, 1983a, b). The E1A region can also transform rodent cells completely in cooperation with other oncogenes (Jochemsen et al., 1986; Pozzatti et al., 1986; Ruley, 1983; Zerler et al., 1986). It has been reported recently that the Ad type 12 (Ad12) E1A region alone confers a tumorigenic phenotype on transformed cells (Gallimore et al., 1985; Kelekar & Cole, 1986). Second, the E1A products of human Ads show multiple functions in eukaryotic cells; they alter the cell cycle of the cells which contain them (Bellett et al., 1985; Braithwaite et al., 1983; Rossini et al., 1979), regulate other early genes of Ads (Jones & Shenk, 1979; Nevins, 1981) and enhance (Gaynor et al., 1984; Kao & Nevins, 1983; Roberts et al., 1985; Stein & Ziff, 1984; Treisman et al., 1983) or repress (Borrrelli et al., 1984; Hen et al., 1985; Schrier et al., 1983) the expression of cellular and viral genes. In addition, recent indirect evidence that there is a cellular E1A-like factor(s) in undifferentiated cells (Hen et al., 1986; Imperiale et al., 1984; Jones, 1986) has attracted attention to the roles these factors might play in the development and gene regulation of eukaryotic cells.
In contrast, little is known about the E1 region of animal Ads (Igarashi et al., 1978; Kimelman et al., 1985; Yamashita et al., 1985). Recently, it was reported that an internal fragment (17.2 to 28.7%) of canine Ad1 [infectious canine hepatitis virus (ICHV)] can transform primary rodent cells as well as the leftmost fragment (0 to 17.2%) (Yamashita et al., 1985). Whether this is common in canine Ads is left unresolved.

We report here on the localization and transforming activities of genes in the E1 region of canine Ad2 (CAd2).

METHODS

Viruses, viral DNA and cells. CAd2 (Tront A 26-61 strain) was propagated in MDCK cells which were maintained in Eagle's MEM supplemented with 1% calf serum. Viral DNA was prepared from infected cells as previously described (Shinagawa et al., 1983). Rat 3Y1 cells (Kimura et al., 1975) were cultured in Dulbecco's modifed MEM (DMEM) supplemented with 10% foetal calf serum (FCS). All the transformed cells were cultured in Eagle's MEM with 5% calf serum. Primary baby rat kidney (BRK) cells were prepared as described (Yamashita et al., 1985).

Molecular cloning of CAd2 DNA fragments. For transfection experiments, CAd2 DNA fragments were cloned into plasmids pUC13 or pUC18. For cloning of a CAd2 terminal fragment, the terminal protein, which is covalently bound to the terminus of the Ad genome, was removed by incubating the terminal fragment at 37 °C for 2 to 3 h in 0.5 m-piperidine. Piperidine was removed by lyophilizing twice and subjecting to ethanol precipitation once. The fragment was annealed by heating at 100 °C and cooling down slowly in 1 m-NaCl, and ligated to the HindIII sites and other appropriate restriction sites of the vectors. To obtain a cloned fragment lacking the left-hand region, partial digestion of the EcoRI C fragment with HindIII was performed. For preparation of single-stranded DNA used in some probes, DNA fragments were cloned into pUC18.

Assays of transforming activity. 3Y1 cells were seeded 2 days before assay to obtain subconfluent growing cells. They were freshly fed 4 h before transfection and were transfected with CAd2 DNA or its fragments by the calcium phosphate method (Graham & van der Eb, 1973). Five to 10 μg DNA was used per 60 mm dish. For cloning, transformed foci were isolated using Pasteur pipettes and transferred to new dishes. For counting of the number of foci, the cells in the dishes were stained with Giemsa stain 40 to 60 days post-transfection.

Preparation of cytoplasmic RNAs and hybridization. Cytoplasmic RNAs were prepared from transformed cells and CAd2-infected MDCK cells 7 h post-infection. Cells were washed twice in phosphate-buffered saline, lysed by suspending in 0.01 m-Tris–HCl pH 7.5, 0.01 m-NaCl, 1.5 mm-MgCl2, 0.5% Nonidet P40 and incubated on ice for 10 min. Nuclei were removed by centrifugation at 800 g for 5 min and an equal volume of 0.02 m-Tris–HCl pH 7.5, 0.35 m-NaCl, 2% SDS, 0.02 m-EDTA was added to the supernatant. The lysates were extracted once with phenol saturated with 10 mm-Tris, 1 mM-EDTA, pH 8.8, twice with phenol–chloroform–isoamyl alcohol (25:24:1), and cytoplasmic RNAs were precipitated by adding 2.5 vol. of ethanol and 0.1 vol. of 3 m-sodium acetate pH 5.2.

Poly(A)+ RNAs were selected by using a column of oligo(dT)-cellulose (Collaborative Research, Waltham, Mass., U.S.A.). The cytoplasmic RNAs were dissolved in 0.5 m-NaCl, 0.5% SDS, 0.01 m-Tris–HCl pH 7.5, 1 mM-EDTA and applied to the column. After washing of the column with 0.1 m-NaCl, 0.05% SDS, 0.01 m-Tris–HCl pH 7.5, 1 mM-EDTA, poly(A)+ RNAs were eluted with the washing buffer without NaCl. They were denatured by heating at 65 °C for 10 min in 50% (v/v) formamide, 6% formaldehyde, 1 × E buffer (0.018 m-Na2HPO4, 0.002 m-NaH2PO4, 0.002 m-NaCl, 0.02 mM-EDTA, 10% glycerol, 0.2 mM-EDTA, 0.1% bromophenol blue just before electrophoresis. Electrophoresis of the RNAs was carried out in 1% agarose gels containing 6% formaldehyde and 1 × E buffer. The buffer for electrophoresis was 1 × E buffer containing 3% formaldehyde. Following electrophoresis the RNAs were transferred to nitrocellulose filters and then immobilized by baking at 80 °C for 2 h.

Prehybridization of the filters was carried out for 20 to 24 h at 42 °C in 50% formamide, 5 × SSC (1 × SSC is 0.15 m-NaCl, 0.015 m-sodium citrate pH 7.4), 1% SDS, 50 mm-sodium phosphate pH 6.5, 1% glycine, 1 × Denhardt's solution (0.02% w/v each of Ficoll, bovine serum albumin and polyvinylpyrrolidone) and 250 μg/ml sonicated calf thymus DNA (CT DNA). Hybridization with 32P-labelled probes was carried out in the abovementioned buffer with 100 μg/ml CT DNA. The probes were constructed by the oligo-labelling method (Feinberg & Vogelstein, 1983, 1984). DNA fragments (20 to 30 ng) were denatured by boiling for 10 min and the probes were synthesized by adding the Klenow fragment of DNA polymerase I, [α-32P]dGTP, three unlabelled dNTPs and random hexanucleotide primers (Pharmacia).

Growth of transformed cells. The medium of the subconfluent cultures was changed to DMEM supplemented with either 10% or 2% FCS 24 h before trypsinization. The trypsinized cells were suspended in DMEM supplemented with 10% or 2% FCS and seeded at 1 × 105 per 60 mm dish. At 24 h intervals, the cells were harvested and counted.

Growth in soft agar. The cells were suspended in DMEM supplemented with 10% FCS containing 0.25% agarose. The cell suspensions were layered on solidified 0.5% agarose medium at 1 × 105 cells/dish.
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Table 1. CAd2 DNA, CAd2 DNA fragments and plasmids harbouring CAd2 DNA fragments, and their transforming activity

<table>
<thead>
<tr>
<th>DNA fragment or plasmid</th>
<th>Region of the genome (%)</th>
<th>Size (bp)</th>
<th>Vectors</th>
<th>Transforming activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAd2 whole</td>
<td>0 to 100</td>
<td>31,920†</td>
<td>–</td>
<td>67/18</td>
</tr>
<tr>
<td>EcoRI A</td>
<td>36.4 to 100</td>
<td>21,500†</td>
<td>–</td>
<td>0/11</td>
</tr>
<tr>
<td>BamHI A</td>
<td>0 to 47.0</td>
<td>15,000</td>
<td>–</td>
<td>9/8</td>
</tr>
<tr>
<td>pUCXD</td>
<td>0 to 15.2</td>
<td>4,860</td>
<td>pUC18</td>
<td>27/12</td>
</tr>
<tr>
<td>pUCEC</td>
<td>0 to 11.3</td>
<td>3,609‡</td>
<td>pUC13</td>
<td>6/12</td>
</tr>
<tr>
<td>pUCPA</td>
<td>0 to 9.1</td>
<td>2,895‡</td>
<td>pUC13</td>
<td>0/19</td>
</tr>
<tr>
<td>pUCPH2</td>
<td>0 to 6.3</td>
<td>2,006‡</td>
<td>pUC13</td>
<td>0/15</td>
</tr>
<tr>
<td>pUCPP</td>
<td>0 to 5.2</td>
<td>1,701‡</td>
<td>pUC13</td>
<td>0/5</td>
</tr>
<tr>
<td>pUCPH</td>
<td>0 to 4.5</td>
<td>1,429‡</td>
<td>pUC13</td>
<td>0/8</td>
</tr>
<tr>
<td>pUCHE</td>
<td>4.5 to 11.3</td>
<td>2,180‡</td>
<td>pUC13</td>
<td>0/11</td>
</tr>
<tr>
<td>pUCEX</td>
<td>11.3 to 32.9</td>
<td>6,900</td>
<td>pUC18</td>
<td>0/10 (0/17)§</td>
</tr>
<tr>
<td>pUCXC</td>
<td>15.2 to 32.9</td>
<td>5,660</td>
<td>pUC13</td>
<td>0/10 (0/9)§</td>
</tr>
<tr>
<td>pUCHfC</td>
<td>0 to 2.1</td>
<td>6,83‡</td>
<td>pUC118</td>
<td>NT</td>
</tr>
<tr>
<td>pUChdC</td>
<td>4.5 to 6.3</td>
<td>577‡</td>
<td>pUC118</td>
<td>NT</td>
</tr>
</tbody>
</table>

* Number of 3Y1 transformed foci/number of dishes examined.
† Sizes of CAd2 DNA and the EcoRI A fragment were from Shinagawa et al. (1987), and those of the others larger than 11.3% were estimated by using size markers.
‡ Sizes of the fragments were estimated from DNA sequence data of the EcoRI C fragment (R. Shibata et al., unpublished data).
§ Numbers in parentheses indicate the results obtained from primary BRK cells.
|| NT, not tested.

RESULTS

Restriction mapping and cloning of CAd2 DNA

In order to localize transforming genes in the CAd2 genome, restriction maps were constructed (Fig. 1a). As was previously reported, EcoRI yielded three fragments and the A and C fragments were the terminal ones (Shinagawa et al., 1983). XbaI yielded four fragments, and BamHI seven. Cleavage sites for XbaI and the location of the BamHI A fragment were determined by Southern hybridization (Southern, 1975), and the double digestion and partial digestion method. For detailed analysis of the left-hand region, cleavage sites in the EcoRI C fragment were mapped for HindIII, PvuII and AccI (Fig. 1b).

For transfection experiments and construction of hybridization probes, it is desirable to use cloned DNA fragments to avoid contamination with other fragments. Fragments from the left-hand region of CAd2 genome were therefore cloned, as summarized in Table 1.
Fig. 2. Morphology of transformed cells. The cells were fixed with methanol and stained with Giemsa stain when confluent. (a) Normal 3Y1 cells; (b) 3Y1EC cells; (c) 3Y1XD cells; (d) 3Y1BA cells; (e) 3Y1CAd2 cells. Bar marker represents 25 μm.

Transforming activity of CAd2 DNA fragments

The transforming activity of CAd2 DNA fragments was assayed using rat 3Y1 cells (Table 1). At first, we used the EcoRI A (36-4 to 100%) and BamHI A (0 to 47-0%) fragments, which represent each of the genome termini (Fig. 1a), to determine the approximate location of the transforming genes. BamHI A showed transforming activity whereas EcoRI A did not, so we determined the former to be the left-hand fragment. As the transforming genes of human Ads (E1A and E1B) have been shown to be in the leftmost 11% of the genome, we tested the activity of cloned fragments smaller than BamHI A. pUCXD (0 to 15-2%) and pUCEC (0 to 11-3%) showed transforming activities in repeated experiments. The plasmids containing the leftmost 9-1% (pUCPA), 6-3% (pUCPH2), 5-3% (pUCPP) and 4-5% (pUCPH) of the genome showed no transforming activity. To test whether the left extremity of the genome is required for transformation, we examined the activity of pUCHE (4-5 to 11-3%). This fragment caused no focus formation, demonstrating a requirement for the left-hand region. From these
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observations, we concluded that the smallest restriction fragment containing transforming activity is *EcoRI* C (0 to 11.3%). As an internal fragment (17.2 to 28.7%) of ICHV has been reported to transform primary cultured BRK cells (Yamashita et al., 1985), we tested pUCEX (11.3 to 32.9%) and pUCXC (15.2 to 32.9%) on 3Y1 and primary BRK cells. No activity was detected in these fragments in our assay.

Transformed cell lines were established from each single focus caused by transfection with the whole DNA, *BamHI* A, pUCXD and pUCEC, and named 3Y1CAd2, 3Y1BA, 3Y1XD and 3Y1EC. In the case of 3Y1XD and 3Y1EC, four and three independent cell lines respectively were established.

**Morphology of transformed cells**

The transformed cells and normal 3Y1 cells were examined morphologically after staining with Giemsa stain (Fig. 2). Normal 3Y1 cells had large cytoplasmic areas and were contact-inhibited when grown to saturation (Fig. 2a). Cells of all the transformed lines, 3Y1EC, 3Y1XD, 3Y1BA and 3Y1CAd2, were smaller than normal 3Y1 cells and the latter three cell lines totally lacked contact inhibition.

**Growth characteristics of transformed cells**

To test serum dependence, the transformed cells were cultured in medium supplemented with either 10% or 2% FCS. All the cells grew well in the medium containing 10% FCS. Medium with 2% FCS supported the growth of 3Y1CAd2 and 3Y1BA cells, but normal 3Y1 cells grew inefficiently, and 3Y1XD and 3Y1EC cells did not grow at all (Fig. 3). This led to the conclusion that 3Y1XD cells and 3Y1EC cells were serum-dependent. Growth in medium containing 1% FCS was also examined. 3Y1CAd2 cells and 3Y1BA cells grew as well in this medium, showing that they were highly serum-independent (data not shown).
Fig. 4. Growth of transformed cells in soft agar medium. The cells were photographed 10 days after seeding. (a) Normal 3Y1 cells; (b) 3Y1EC cells; (c) 3Y1XD cells; (d) 3Y1BA cells; (e) 3Y1CAd2 cells. Bar marker represents 25 μm.

Table 2. Colony formation by transformed cells in soft agar medium

<table>
<thead>
<tr>
<th>Size of colony (mm)</th>
<th>3Y1CAd2</th>
<th>3Y1BA</th>
<th>3Y1XD</th>
<th>3Y1EC</th>
<th>3Y1</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;0.1</td>
<td>1.0 × 10⁴</td>
<td>2.1 × 10³</td>
<td>1.1 × 10³</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&gt;0.2</td>
<td>1.0 × 10⁵</td>
<td>1.0 × 10²</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Number of colonies per 60 mm dish was counted 3 weeks after seeding with 1 × 10⁵ cells per dish.

We tested anchorage-independent growth of the transformed cells. 3Y1CAd2 cells and 3Y1BA cells grew rapidly in soft agar and formed large colonies which could be seen by the naked eye 3 to 4 weeks after seeding (Table 2 and Fig. 4). 3Y1XD cells grew a little in soft agar, forming smaller colonies than the previous two lines. 3Y1EC cells, as well as 3Y1 cells, grew
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Table 3. Tumorigenicity of transformed cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumorigenicity*</th>
<th>Observation period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3Y1CAD2</td>
<td>4/4</td>
<td>20†</td>
</tr>
<tr>
<td>3Y1BA</td>
<td>5/5</td>
<td>10†</td>
</tr>
<tr>
<td>3Y1XD</td>
<td>0/10</td>
<td>150</td>
</tr>
<tr>
<td>3Y1XD‡</td>
<td>0/10</td>
<td>160</td>
</tr>
<tr>
<td>3Y1EC</td>
<td>0/6</td>
<td>190</td>
</tr>
<tr>
<td>3Y1</td>
<td>0/4</td>
<td>100</td>
</tr>
</tbody>
</table>

* Number of rats with tumours/number of rats injected with 5 x 10^6 cells.
† Tumours were observed in all animals by the time indicated.
‡ An additional independent cell line 3Y1XD was examined.

Table 4. Summary of characteristics of transformed cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Morphological transformation</th>
<th>Growth in soft agar</th>
<th>Serum dependence</th>
<th>Tumorigenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3Y1CAD2</td>
<td>+ (1)</td>
<td>+ (1)</td>
<td>- (1)</td>
<td>+ (1)</td>
</tr>
<tr>
<td>3Y1BA</td>
<td>+ (1)</td>
<td>+ (1)</td>
<td>- (1)</td>
<td>+ (1)</td>
</tr>
<tr>
<td>3Y1XD</td>
<td>+ (4)</td>
<td>± (4)</td>
<td>+ (2)</td>
<td>- (2)</td>
</tr>
<tr>
<td>3Y1EC</td>
<td>+ (3)</td>
<td>- (3)</td>
<td>+ (1)</td>
<td>- (1)</td>
</tr>
<tr>
<td>3Y1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* + indicates that the cells were positive for the characteristic; — indicates that the cells were negative for the characteristic. Numbers in parentheses indicate the number of cell lines tested.

little and formed only small colonies. Three and two independent lines of 3Y1XD and 3Y1EC cells respectively were tested qualitatively and the same results were obtained (data not shown).

Tumorigenicity of transformed cells

The tumorigenicity of the transformed cells in syngeneic rodents was tested using newborn F344 rats. Transformed cells (5 x 10^6/rat) were injected subcutaneously into 3-day-old animals. 3Y1CAD2 cells formed palpable tumours 20 days post-injection (p.i.) in all the rats (Table 3). 3Y1BA cells showed high tumorigenicity; two of the five rats developed tumours 5 days p.i. and all the animals had developed them within 10 days p.i. None of the animals developed tumours when injected with two independent cell lines of 3Y1XD cells and one cell line of 3Y1EC cells within 150, 160 and 190 days respectively of the observation period.

The characteristics of the transformed cells are summarized in Table 4. Based on the results regarding anchorage independence, serum independence and tumorigenicity, it was concluded that 3Y1CAD2 cells and 3Y1BA cells were fully transformed, and 3Y1XD cells and 3Y1EC cells were incompletely transformed.

Analysis of viral RNAs in transformed cells

Cytoplasmic poly(A)^+ RNAs from transformed cells and those of CAd2-infected MDCK cells at 7 h post-infection (expected to be viral early mRNAs) were analysed. When probed with the BamHI A fragment, two major transcripts, about 1 kb and 2 kb, were detected in all the transformed cells (Fig. 5a). The sizes of these RNAs were estimated using restriction fragments of pBR322, which were co-electrophoresed. As corresponding bands were seen in the lane representing early infected cells, it was confirmed that they were the products of the early viral gene(s). Since the 1 kb and the 2 kb transcripts were detected in all the transformants, sequential hybridization was carried out to examine whether they were encoded in independent regions, as are the E1A and E1B of human Ads which are involved in transformation (Bernards et al., 1982; Hurwitz & Chinnadurai, 1985a; Montell et al., 1984; van den Elsen et al., 1982, 1983a, b). Fig. 5(b) shows that the 1 kb RNA was transcribed from the 0 to 4.5% region, and the 2 kb RNA
Fig. 5. Northern blot hybridization analysis of transformed cell RNAs. (a) The BamHI A fragment (0 to 47%) was used as a probe. Poly(A)* RNAs (1 µg, lane 1; 2 µg, lanes 2 to 6) were electrophoresed. Lane 1, early infected cells; lane 2, 3Y1CAd2 cells; lane 3, 3Y1BA cells; lane 4, 3Y1XD cells; lane 5, 3Y1XD cells (another cell line); lane 6, 3Y1EC cells; lane M, size markers (mixture of pBR322 fragments). (b) Sequential hybridization of transformed cell RNAs by the HindIII fragments of EcoRI C. The probes used are shown in the lower part of the figure. Poly(A)* RNAs (4 µg, lanes 1 to 4; 2 µg, lanes 5) were electrophoresed. Lanes 1 and 5, early infected cells; lanes 2, 3Y1CAd2 cells; lanes 3, 3Y1BA cells; lanes 4, 3Y1EC cells; lanes M, size markers (mixture of pUCEC fragments).
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Fig. 6. Northern blot hybridization analysis of transformed cell RNAs. Single-stranded DNA fragments (1 strand; a, 0 to 2.1%; b, 4.5 to 6.2%) were used as probes. Poly(A)+ RNAs (1 μg, lanes 1; 2 μg, lanes 2 to 6) were electrophoresed. Lanes 1, early infected cells; lanes 2, 3Y1CAD2 cells; lanes 3, 3Y1BA cells; lanes 4 and 5, 3Y1XD cells; lanes 6, 3Y1EC cells. The sizes estimated from the migration distance of RNA size markers are indicated.

from the 4.5 to 11.3% region. A very faint band of about 1.1 kb was detected by the 4.5 to 6.3% probe in all the transformants. This transcript could not be detected by other probes because it was not abundant in the transformed cells. As the 1.1 kb and the 2 kb transcripts could not be detected when probed with the 11.3 to 15.2% fragment (data not shown), these genes seem to be encoded within the leftmost 11.3% of the genome. This assumption was supported by nucleotide sequence data (R. Shibata, M. Shinagawa, Y. Iida & T. Tsukiyama, unpublished data). The coding strands of these transcripts were determined using probes (0 to 2.1% and 4.5 to 6.3%) that were synthesized from single-stranded DNAs prepared from fragments cloned in pUC118 (Fig. 6). It was shown that they were encoded on the r strand, as for human Ads. In the lanes representing 3Y1BA and 3Y1XD cells (Fig. 5), there were additional transcripts larger than 2 kb. One such transcript from two independent 3Y1XD cell lines seemed to be the fused product of the 1 kb and the 2 kb RNAs because they were detected by both the 0 to 2.1% and the 4.5 to 6.3% probes (Fig. 6). A similar transcript was also detected as a very faint band (Fig. 6) for 3Y1BA and 3Y1CAD2 (lanes 3 and 2 respectively). The length (3 kb) of this transcript supports this assignment. This type of transcript has been reported previously in Ad12 and Ad7 (Sawada & Fujinaga, 1980; Yoshida & Fujinaga, 1980). As shown in Fig. 6a, lanes 4 and 5) for 3Y1XD cells, additional transcripts were observed as minor components of between 1 kb and 3 kb. We did not examine whether these transcripts were present in other transformants. The additional transcript (larger than 3 kb) of 3Y1BA cells (lanes 3) seems not to be the readthrough product, because this mRNA was detected by the 4.5 to 11.3% probe but not by the 0 to 4.5% probe (Fig. 5b and 6) and because this transcript is larger than 3 kb. Although the origin of this RNA is not completely defined, this additional transcript in 3Y1BA is not considered to be responsible for complete transformation because it was not seen in 3Y1CAD2. When probed by the 15.2 to 32.9% fragment, there were no detectable transcripts in the transformed cells (data not shown).

DISCUSSION

The transfection experiments with CAd2 DNA fragments revealed that the leftmost 11.3% of the genome retained an ability to transform 3Y1 cells. From Northern blot analysis, it seemed
that a 1 kb RNA was transcribed from the 0 to 4.5% region, and 1-1 kb and 2 kb RNAs from the 4.5 to 11.3% region. DNA sequence analysis of this region supported these data (R. Shibata, M. Shinagawa, Y. Iida & T. Tsukiyama, unpublished data), i.e. a 0.8 kb mRNA could be encoded in the 0 to 4.5% region and 0.9 kb and 2.0 kb mRNAs in the 4.5 to 11.3% region. The coding regions of the mRNAs are similar to the E1 genes of human Ads (Grand, 1987). It is possible that the genes encoded in the 0 to 11.3% region of CAd2 act as early genes because they are transcribed very early (7 h) after infection. The transforming function of these genes, however, seems to be dissimilar to that of the E1 genes. In the case of human Ads, only the E1 region can induce a transformed phenotype that is identical to those induced by the whole viral genome (Grand, 1987). The growth characteristics of the cells transformed by the 0 to 15.2% region of CAd2 were different to those transformed by the whole genome or by BamHI A (0 to 47%). The BamHI A fragment or the whole genome of CAd2 induced complete loss of contact inhibition, serum independence, anchorage independence and tumorigenicity of transformed cells, whereas the 0 to 15.2% and 0 to 11.3% regions of CAd2 induced only morphological transformation, and the transformed cells derived by using these regions (3Y1EC and 3Y1XD) seemed to be restricted in their growth in low-serum medium. We have no explanation for this at present. The genes encoded in both the 0 to 4.5% and the 4.5 to 11.3% regions were shown to be required for this activity. This observation suggests that some gene(s), not yet identified, may be required in addition to the 0 to 11.3% region for complete transformation. The gene(s) is thought to be located in the 15.2 to 45%, region or across the 11.3 to 15.2% region; however, we failed to detect transcripts from this region using the 11.3 to 15.2% fragment as the probe. This may have been due to low levels of gene expression.

In recent years, it has been proposed that E1A of human Ads contains three functional domains (reviewed by Moran & Mathews, 1987). From mutational analysis, it was proposed that transformation activity correlates with enhancer repression, not with trans-activation activity (Kuppuswamy & Chinnadurai, 1987; Lillie & Green, 1986). It will be worth investigating the activating and repressing activity of transforming genes of CAd2.

Shiroki et al. (1984) have reported that the expression of the Ad12 E4 gene was required for establishment of soft agar colony-forming rat cell lines. To test whether this was the case in CAd2, poly(A)+ RNAs from infected cells and transformed cells were probed by the 96.4 to 100% fragment. Although distinct transcripts were detected in infected cells, none were detected in transformed cells (data not shown). There is no evidence that these transcripts correspond to the E4 gene products of Ad12; however, the gene(s) on the right of the CAd2 genome seemed not to contribute to the transformation.

From this and a previous report (Yamashita et al., 1985), it appears that two serotypes of canine Ad, ICHV and CAd2, have types of transforming genes different to those of human Ads. Comparative studies of the transforming genes of canine Ads and human Ads will reveal some aspects of the mechanism of transformation.

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Canine Ad2 transforming genes


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