Analysis of Virus-specific mRNAs Present in Cells Transformed with Restriction Fragments of Adenovirus Type 5 DNA

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SUMMARY

Adenovirus type 5 (Ad5) mRNAs present in cells transformed with left-terminal Ad5 DNA fragments (XhoI-C, 0 to 15.5%; HindIII-G, 0 to 7.7%; HpaI-E, 0 to 4.3%) were characterized by ‘Northern blotting’ and S1 nuclease analysis. They were compared with the mRNAs transcribed from the Ad5 E1 region in the early and late stages of lytic infection. It is shown that in XhoI-C-transformed cells the same mRNAs were transcribed as early during lytic infection: two co-terminal mRNAs from region Ela, differing only in their splicing, and one major Elb transcript. In HindIII-G-transformed cells additional Ela mRNAs were detected with a novel 5' terminus, but with the normal splicing pattern. Instead of the normal Elb mRNA, HindIII-G-transformed cells were found to contain mRNAs consisting of a viral Elb segment and a non-viral segment. This Elb-encoded segment was shown not to be involved in RNA splicing. The mRNAs in cells transformed with Ad5 HpaI-E were similar to the Ela mRNAs found in XhoI-C- and HindIII-G-transformed, and in lytically infected cells but had aberrant 3' termini. These results are discussed in the light of the Ad5 E1 DNA and RNA sequences, and protein mapping data.

INTRODUCTION

Previous studies have shown that transformation of cells by human adenoviruses (Ad) is a process in which only a small portion of the viral genome is involved (Gallimore et al., 1974; Graham et al., 1974). It has been well established that this transforming activity is located at the left end of the linear Ad genome of 36 500 base pairs, in a segment comprising the early region 1 (E1) which extends from 1.3 to 11.2% (Van der Eb et al., 1979).

Recent studies by a number of laboratories have shown that the E1 region consists of two parts, subregions Ela (1.3 to 4.5%) and Elb (4.7 to 11.2%), each coding for a family of co-terminal mRNAs differing in their splicing patterns (Berk & Sharp, 1977; Chow et al., 1979; Perricaudet et al., 1979, 1980). From RNA mapping data and DNA sequence analysis, it has become evident that each subregion has its own promoter and polyadenylation signal (Van Ormondt et al., 1980). DNA transfection studies have shown that not only the intact E1 region but also parts of it have transforming activity (Van der Eb et al., 1979). So far, the smallest Ad type 5 DNA fragment known to transform cells in vitro is the HpaI-E fragment (map units 0 to 4.3%, Houweling et al., 1980).

The fragment-transformed cells were found to differ phenotypically, depending on the length of the Ad DNA segment used for transformation, and could be divided into three classes.

I. Cells transformed with DNA fragments containing the entire E1 region (e.g. XhoI-C, 0 to 15.5%); in cell morphology, growth properties etc., these cells are indistinguishable from those transformed with intact viral DNA or with virions, and can induce tumours in nude mice.

II. Cells transformed with DNA fragments lacking part of the Elb but containing the entire Ela subregion (e.g. HindIII-G, 0 to 7.7%); these cells morphologically resemble class I-transformed cells but differ from these by having an atypical T antigen staining pattern and...
slightly different growth properties. Furthermore, class II cells are unable to induce tumours in nude mice.

III. Cells transformed with DNA fragments comprising subregion E1a only (e.g. HpaI-E, 0 to 4.3%) ; these cells clearly differ from class I cells in that they have a more or less fibroblastic rather than an epithelial appearance and are unable to reach high or even intermediate saturation densities. Class III cells are not tumourigenic in nude mice (Van der Eb et al., 1979; P. J. van den Elsen, P. I. Schrier, A. Houweling, B. S. de Pater, J. van der Veer & A. van der Eb, unpublished results).

An analysis of the proteins found in the various classes of transformed cells revealed that class II cells differ from class I cells by the absence of the major 55 kdal (kilodalton) T antigen and a number of minor antigens whereas class III cells lack the 21 kdal T antigen as well (Schrier et al., 1979; P. J. van den Elsen et al., unpublished results). The molecular weights of the two major E1b polypeptides of Ad5 are 21 kdal and 55 kdal, based on the nucleotide sequence (Bos et al., 1981). However, after immunoprecipitation and depending on the gel system and mol. wt. markers used, polypeptides usually of 19 kdal and 65 kdal are detected.

We have embarked upon a study of the expression of the viral information present in class I, II and III cells at the level of transcription. To this end, we have characterized the virus-specific RNAs by means of Northern blotting and S1 nuclease analysis with viral DNA probes. In the present paper we show that when the viral polyadenylation signal is absent in the transformed cells, discrete RNA species are synthesized which differ from the RNAs usually transcribed from region E1 and which vary in size from one cell line to the other.

### METHODS

**Cells, viruses and viral DNA.** KB cells were grown in suspension or as monolayer cultures with Eagle’s minimal essential medium (MEM) supplemented with 5% heat-inactivated horse serum, or 8% newborn calf serum respectively. Adenovirus type 5 (strain ‘Adenoid 75’) was obtained from the American Type Culture Collection and was grown in KB cells at a multiplicity of infection of 20. Virus was purified from infected cells and viral DNA was isolated as described previously (Van der Eb et al., 1969). Transformed cells were grown in MEM supplemented with 8% newborn calf serum.

**Isolation of cytoplasmic RNA.** For preparation of early or late cytoplasmic viral RNA, KB cells in spinner cultures were infected with a freshly prepared virus stock at 100 to 200 p.f.u. or 20 p.f.u. per cell respectively. Cytoplasmic RNA was isolated from monolayer cultures of transformed cells. After harvesting, the cells were washed 3 or 4 times with ice-cold isotonic high pH buffer (0.14 M NaCl, 0.001 M MgCl2 and 0.01 M Tris-HCl pH 8.5) and lysed for 10 min in ice by resuspending the cells in 10 vol. of isotonic high pH buffer containing 0.5% Nonidet P40 and 40 μg/ml dextran sulphate (Büttner et al., 1974). The lysate was centrifuged to remove the nuclei and the nuclear pellet was extracted once more with an equal volume of lyss buffer. SDS, EDTA and NaCl were added to the combined supernatants to final concentrations of 1%, 0.05 M and 0.3 M respectively, followed by three extractions with an equal volume of a 1:1 mixture of buffer-saturated phenol and chloroform/isoamyl alcohol (24:1). Total RNA was precipitated with 2 vol. 96% ethanol at −25 °C. Poly(A)-containing RNA was isolated from the preparation of total RNA by selective retention on oligo(dT)-cellulose (Aviv & Leder, 1972).

**Analysis of cytoplasmic RNA by means of Northern blotting.** A 50 μg amount of total cytoplasmic RNA or poly(A)-containing RNA was fractionated by electrophoresis in vertical slab gels containing 1.2% agarose and 5 mm methylmercuric hydroxide as the denaturing agent according to Bailey & Davidson (1976). Electrophoresis was carried out at 40 V for 16 to 18 h at room temperature. Gel transfer and preparation of DBM paper were as described by Alwine et al. (1977). Virus-specific sequences were detected by hybridization with 32P-labelled DNA fragments (Wahl et al., 1979).

**S1 mapping of cytoplasmic RNAs.** Hybridizations were performed in 30 μl of S1 hybridization mixture at 53 °C for 16 to 18 h according to Berk & Sharp (1978). In the hybridization mixture poly(A)-containing RNA was present at a concentration of 200 μg/ml and the 32P-labelled DNA fragment at 5 μg genome equivalent/ml. The RNA/DNA hybrids were treated with 400 units/ml of S1 nuclease for 30 min at 37 °C and were analysed on 2% alkaline gels according to McDonnell et al. (1977). The adenovirus DNA fragments were labelled with 32P by nick translation prior to hybridization. The single-strand nicks were repaired with T4 DNA ligase. Alternatively, in some cases unlabelled DNA fragments were hybridized to poly(A)-containing RNA. The S1-resistant material was fractionated in 2% alkaline agarose slab gels and blotted onto nitrocellulose paper (Southern, 1975). Virus-specific sequences were detected by hybridization with 32P-labelled DNA fragments (Wahl et al., 1979).

**Materials.** Methylmercuric hydroxide was obtained as a 1 M stock solution from Alpha Chemicals. α-32P-labelled deoxyribonucleoside triphosphates were obtained from Amersham International. DNA polymerase I and...
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Fig. 1. Left-hand end of the adenovirus type 5 genome showing the positions of the early regions E1a and E1b, the cleavage sites for several restriction endonucleases and the positions of the DNA fragments used for the hybridization experiments.

Table 1. RNA species detected by Northern blot analysis in BRK cells transformed with left-terminal Ad5 DNA fragments and in KB cells lytically infected with Ad5

<table>
<thead>
<tr>
<th>RNA species detected (kb)</th>
<th>BRK cells transformed by Ad5 segment</th>
<th>KB cells lytically infected</th>
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<tbody>
<tr>
<td></td>
<td>XhoI-C (0-15.5%)</td>
<td>HindIII-G (0-7.7%)</td>
</tr>
<tr>
<td></td>
<td>E1a</td>
<td>E1a</td>
</tr>
<tr>
<td></td>
<td>Elb</td>
<td>Elb</td>
</tr>
<tr>
<td>2.5</td>
<td></td>
<td>1.25</td>
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<tr>
<td>1.0</td>
<td></td>
<td>1.15</td>
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<td>1.5</td>
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<td>1.0</td>
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<td>1.5</td>
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<td>0.7</td>
</tr>
</tbody>
</table>

* E1a mRNAs detected in the HpaI-E fragment-transformed cells: clone 1, 1.15-1.5 kb, 1.6 kb, 2.15 kb and 3.2 kb; clone 7, 1.6 kb and 2.5 kb.
† E1b mRNAs detected in HindIII-G fragment-transformed cells: clone 5, 1.5 kb; clone 6 acl, 1.15 kb and 1.25 kb.

pancreatic DNase were purchased from Boehringer Mannheim. S1 nuclease and agarose were from Sigma. T4 DNA ligase, prepared from a lysogen of λ T4lig (Murray et al., 1979), with an additional purification step on hydroxylapatite, was a gift of Dr A. de Waard.

RESULTS

Virus-specific mRNAs isolated from DNA fragment-transformed cells were analysed by Northern blotting and S1 nuclease analysis, and were compared to the region E1 mRNAs generated both early and late during lytic infection. Fig. 1 shows the map positions of the DNA fragments used as probes in this study.

Northern blotting

The results of several RNA blotting experiments are summarized in Table 1. A few illustrative cases will be described in detail.

Total or poly(A)-containing RNA from DNA fragment-transformed cells was fractionated in 1.2% agarose slab gels containing 5 mM-methylmercuric hydroxide as the denaturing agent. The fractionated RNAs were blotted onto DBM paper as described in Methods, and virus-specific RNAs were identified by hybridization with 32P-labelled Ad5 HpaI-E fragment representing subregion E1a and with HpaI-C which contains all of subregion E1b (Fig. 1).

As is evident from Fig. 2, cells transformed by Ad5 XhoI-C (0 to 15.5%) contained two E1a-specific RNAs which were 1.15 kb (kilobases) and 1.0 kb long (Fig. 2, lane 2) and one E1b-specific RNA of 2.5 kb (lane 8). A particular cell line transformed by Ad5 HindIII-G (0 to 7.7%) contained, in addition to the 1.15 kb and 1.0 kb E1a mRNAs, and RNA of 1.25 kb (Fig. 2, lane 3), and E1b-specific RNAs of 1.15 kb and 1.25 kb (lane 9). Similar experiments with another HindIII-G-transformed cell line yielded the same E1a-specific products (Fig. 2, lane 4); this cell
Fig. 2. Identification of virus-specific mRNAs present in cytoplasmic extracts from DNA fragment-transformed cells. Total cytoplasmic RNA from XhoI-C-transformed cells and poly(A)-selected RNA from HindIII-G- and HpaI-E-transformed cells were compared with total early RNA from lytically infected KB cells. The RNAs were fractionated in a 1.2% agarose denaturing slab gel and, after electrophoresis, blotted onto DBM paper (see Methods). Lanes 1 to 6: virus-specific RNA was detected by hybridization with $^{32}$P-labelled HpaI-E (E1a probe). Lanes 7 to 10: RNA was detected by hybridization with $^{32}$P-labelled HpaI-C (E1b probe). Lanes 1 and 7, early RNA; lanes 2 and 8, XhoI-C (clone 4) RNA; lanes 3 and 9, HindIII-G (clone 6 act) RNA; lanes 4 and 10, HindIII-G (clone 5) RNA; lanes 5 and 6, HpaI-E RNA (clones 1 and 7 respectively).

Fig. 3. Identification by Northern blotting of virus-specific RNAs present at early and late times in lytic infection (see also the legend to Fig. 2). Lanes 1, 3 and 5: total early RNA (6 h). Lanes 2, 4 and 6: total late RNA (24 h). Virus-specific RNA was detected with $^{32}$P-labelled HpaI-E (0 to 4.3%), lanes 1 and 2), $^{32}$P-labelled HpaI-C (4.3 to 25.5%, lanes 3 and 4) and with $^{32}$P-labelled SmaI-F (2.8 to 10.7%, lanes 5 and 6). The 1.6 kb band that is apparently present in both lanes 2 and 6 is due to the effect of large amounts of 18S ribosomal RNA migrating at the same position in the gel.

line, however, contained a major E1b-specific RNA of different length (lane 10). None of these lines, however, contained the 2.5 kb E1b species found in XhoI-C-transformed cells. Different cell lines obtained by transformation of primary cells with Ad5 HpaI-E (0 to 4.3%) as expected contained no RNA material hybridizing to the E1b probe, whereas the E1a-specific RNAs had
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Table 2. Ad5 DNA segments rendered resistant to nuclease S1 digestion after hybridization with RNA from cells transformed or lytically infected by Ad5

<table>
<thead>
<tr>
<th>DNA fragment used for S1 analyses</th>
<th>BRK cells transformed by Ad5 fragment</th>
<th>Lytic infection</th>
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<tbody>
<tr>
<td></td>
<td>XhoI-C (0-15.5%)</td>
<td>HindIII-G (0-7.7%)</td>
</tr>
<tr>
<td>HpaI-E (0-4.3%)</td>
<td>–</td>
<td>975</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>835</td>
</tr>
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<td></td>
<td>640</td>
<td>640</td>
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<td>500</td>
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<tr>
<td></td>
<td>340</td>
<td>340</td>
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<td>–</td>
<td>–</td>
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<tr>
<td>HindIII-G (0-7.7%)</td>
<td>1100</td>
<td>1100</td>
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<tr>
<td></td>
<td>–</td>
<td>975</td>
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<td>500</td>
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<tr>
<td></td>
<td>395</td>
<td>395</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HpaI/HindIII (4-3-7-7%)</td>
<td>1100</td>
<td>1100</td>
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<tr>
<td></td>
<td>–</td>
<td>–</td>
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<tr>
<td>HindIII-E (7-7-17%)</td>
<td>660</td>
<td>ND</td>
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<tr>
<td></td>
<td>450</td>
<td>–</td>
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<td>Smal-F (2-8-10-7%)</td>
<td>1850</td>
<td>–</td>
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<td></td>
<td>–</td>
<td>1100</td>
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<td>105</td>
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<tr>
<td>Mbol (0-1-7%)</td>
<td>–</td>
<td>490</td>
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<tr>
<td></td>
<td>125</td>
<td>125</td>
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</table>

* ND, Not done.

lengths varying from one cell line to the other. Fig. 2 lanes 5 and 6 show the electrophoretic behaviour of the E1α-specific RNAs extracted from two particular HpaI-E-transformed cell lines.

Northern blots prepared from RNA isolated early and late after the onset of lytic infection of KB cells are presented in Fig. 2 and 3. In the early stage of infection two mRNAs, 1-0 kb and 1.15 kb long, were detected by the E1α probe, while later in the infectious cycle additional E1α-specific RNAs of 0.7 kb and 2.15 kb were shown to be present (Fig. 3, lanes 1 and 2). The major early E1b mRNA was 2.5 kb long; later during infection new species of E1b-specific RNAs of 0.7 kb and 1.15 kb were generated in large quantities (Fig. 3, lanes 4 and 6). The 1.15 kb E1b mRNA was observed occasionally as early as 6 h after infection but in minute and variable amounts. In Fig. 3, lane 4, it can be seen that the Ad5 HpaI-C probe hybridized to a late 1.6 kb RNA; this probably is the messenger for viral polypeptide IVa2 (map coordinates 16.0 to 11.2%; Van Beveren et al., 1981) since it was absent early in infection and, when present late, did not hybridize to Smal-F (2-8 to 10.7%). The 2.15 kb late RNA species, which hybridized with the E1α probe (Fig. 3, lane 2), was probably also detected with the E1b probes (Fig. 3, lanes 4 and 6). The Ad5 early mRNAs closely resembled those transcribed from the corresponding region of the related adenovirus type 2 (Ad2) (Berk & Sharp, 1977, 1978; Chow et al., 1979; Perricaudet et al., 1979, 1980).

Comparison of the E1 RNA species observed in DNA fragment-transformed and lytically infected cells shows that the XhoI-C-transformed cells contained the E1 mRNAs also detected early during infection, that in HindIII-G-transformed cells the early E1b RNA of 2.5 kb was replaced by species of variable length, and that in HpaI-E-transformed cells also the E1α-specific RNAs had variable length.
Fig. 4. Nuclease S1 analysis of mRNAs present in DNA fragment-transformed cells and in the early and late phases of lytic infection. (a) Segments of $^{32}$P-labelled HpaI-E protected by early RNA (lane 2), late RNA (lane 3), RNA from cells transformed by XhoI-C clones 1 and 4 (lanes 4 and 5 respectively), RNA from cells transformed by HindIII-G clones 5 and 6 acl (lanes 6 and 7 respectively) and in the absence of RNA (lane 8). The 1572-nucleotide S1-resistant tract present in lanes 2 to 8 represents self-annealed input $^{32}$P-labelled HpaI-E. Lanes 1 and 9, $^{32}$P-labelled pBR322 digested with HinfI (size markers). (b) An alternative method for identification of RNA species in a HpaI-E-transformed line. Unlabelled fragment HpaI-E was hybridized to transformed-cell RNA, and S1-resistant DNA tracts were fractionated and blotted onto nitrocellulose paper. DNA was detected by hybridization with $^{32}$P-labelled HpaI-E. Lane 1, DNA from cells transformed by HpaI-E (clone 1); lane 2, late RNA; lane 3, no RNA. The 130-nucleotide S1-resistant tract of HpaI-E that is detected in late RNA is too small to bind to nitrocellulose and therefore was not detected.

S1 nuclease analysis

The mRNAs were further characterized by S1 nuclease analysis (Berk & Sharp, 1978). Poly(A)-containing RNA from fragment-transformed and from lytically infected cells was hybridized to various Ad5 DNA fragments which had been $^{32}$P-labelled by nick translation (see Fig. 1). The mRNA–DNA hybrids were treated with the single-strand-specific nuclease S1, and the resistant material was fractionated in alkaline gels as described in Methods. The results of the S1 nuclease analysis are summarized in Table 2, and some illustrative experiments are shown in Fig. 4, 5, 6 and 7.

To characterize E1a-specific transcripts poly(A)-containing RNA was hybridized to $^{32}$P-labelled or unlabelled Ad5 HpaI-E (0 to 4.3%$^\text{a}$; Fig. 4a and b respectively). Three nuclease S1-resistant tracts with sizes of 640, 500, and 340 nucleotides (N) were observed early during infection (Fig. 4a, lane 2), whereas at late times an additional tract of 130 N appeared (Fig. 4a, lane 3). In XhoI-C- and HindIII-G-transformed cells the E1a mRNAs were the same as those observed in the early stages of infection except that in HindIII-G-transformed cell lines additional E1a-specific tracts of 975 N and 835 N were detected (Fig. 4a, lanes 4, 5, 6 and 7). This 835 N tract was also observed in HpaI-E-transformed cells, together with the 640 N species found in lytically infected cells (Fig. 4b), but the 500 N and 340 N tracts were present in very low amounts in HpaI-E transformed cells (Fig. 4b, lanes 1 and 2).

In order to characterize the E1b-specific mRNAs, poly(A)-containing RNA was hybridized to either $^{32}$P-labelled Ad5 HpaI/HindIII (4.3 to 7.7%$^\text{a}$), Ad5 Smal-F (2.8 to 10.7%$^\text{a}$) or Ad5 HindIII-E (7.7 to 17.0%$^\text{a}$). Fig. 5 shows that the Ad5 HpaI/HindIII fragment (4.3 to 7.7%$^\text{a}$) yielded S1-resistant tracts of 1100 N after hybridizing to RNA from HindIII-G- and XhoI-C-
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Fig. 5. Nuclease S1-resistant tracts of 32P-labelled HpaI/HindIII (4.3 to 7.7%) after hybridization with RNA from cells transformed by the Ad5 fragments HindIII-G (clones 6 acl and 5, lanes 3 and 4 respectively) and XhoI-C (clones 4 and 1, lanes 5 and 6 respectively), with RNA from lytically infected cells (late and early; lanes 7 and 8 respectively) and in the absence of RNA (lane 2). Lane 9, 32P-labelled HpaI/HindIII; lane 1, 32P-labelled pBR322 digested with HindIII (size markers).

transformed cells and RNA from lytically infected cells (early and late) (Fig. 5, lanes 3, 4, 5, 6, 7 and 8). Furthermore, an additional S1-resistant tract of 560 N was present after hybridization to late RNA (lane 7). Fig. 6 shows that Ad5 SmaI-F yielded S1-resistant tracts of 1850 N, 395 N, 355 N and 105 N after hybridization to RNA from lytically infected (early and late) and XhoI-C-transformed cells (Fig. 5, lanes 2, 3 and 4), and an additional 560 N product after annealing to late RNA (lane 3). RNA from a HindIII-G-transformed cell line did not yield the 1850 N and 355 N species, but did give rise to a new 1100 N tract.

The segments rendered resistant to nuclease S1 attack by Ad5 HindIII-E (7.7 to 17.0%) were 660 N and 450 N long, when lytically infected at an early stage of infection or XhoI-C-transformed cells were the source of the RNA (Fig. 7, lanes 4, 5 and 7). RNA isolated late after infection yielded additional tracts of 1400 N and 120 N (lane 6) which represented material hybridizing to the semi-late mRNA from viral protein IVa2; this mRNA has been mapped between positions 5836 (5' end) and 4060 (3' end) of the Ad5 DNA sequence (Van Beveren et al., 1981).

DISCUSSION

In this paper we describe a comparison, by Northern blotting and S1 nuclease analysis, of the RNAs transcribed from viral DNA sequences present in cells transformed by Ad5 DNA fragments of different lengths. As stated in Introduction, such cells can be grouped into three phenotype classes, depending on whether they contain (I) the intact Ad5 E1 region (i.e. the
intact Ela and E1b subregions, e.g. Ad5 XhoI-C), (II) intact subregion Ela plus about half of subregion E1b (e.g. HindIII-G), or (III) only subregion E1a (e.g. HpaI-E).

The RNAs found in representatives of the three classes were compared with the messenger RNAs transcribed from the Ad5 Ela and E1b subregions in cells that had been lytically infected with Ad5 virions. Since Ad5 mRNA formation is a temporally controlled process, we examined the E1 mRNAs present in infected cells both in the early (6 h) and late (24 h) stages of the infectious cycle (Fig. 8).

In lytic infection, the Ad5 E1a subregion codes for a family of 5′- and 3′-co-terminal mRNAs of 1.15 kb, 1.0 kb and 0.7 kb which differ only in the amount of internal sequences removed by RNA splicing. Of these three mRNAs, the 0.7 kb mRNA is an exclusively late species. It is not present in class I, II and III transformed cells. We were unable to map by S1 nuclease analysis the 2.15 kb late species that is detectable in Northern blots after hybridization with the Ela probe and possibly also with the E1b probes (Fig. 3, lanes 2, 4 and 6). The XhoI-C- and HindIII-G-transformed cells contain the 1.15 kb and 1.0 kb RNAs also detected early in lytic infection. Interestingly, the HindIII-G-transformed cells contain additional E1a transcripts which apparently are spliced normally, as judged from the S1 nuclease experiments (Table 2). Protection by RNA of the E1a probe yielded S1-resistant tracts of 975 N and 835 N, in addition to the species observed in lytically infected cells. This probably implies that in HindIII-G-transformed cells, RNA transcription can initiate not only at nucleotide 499, as in infected cells (Baker & Ziff, 1980), but also at sites closer to the left end of the viral genome. Evidence supporting this hypothesis was provided by the primary structure (not shown) of a cloned cDNA
which was derived from E1a mRNA. In this cDNA, nucleotides corresponding to Ad5 DNA residues 974 and 1229 are covalently linked, so that it must be derived from the 1.0 kb RNA or an identically spliced analogue. In the same clone the nucleotide that is adjacent to the oligo(dC) tract, added for cloning purposes, corresponds to Ad5 DNA residue 411. This indicates that the RNA that had served as template for this particular cDNA had the customary cap site (nucleotide 499) at an internal position.

In the HpaI-E-transformed cells, the E1a-specific mRNAs have aberrant sizes (this paper, Table 1; Houweling et al., 1980). Table 2 suggests that they are spliced in the usual way (the 500 N tract being present in low amounts). Protection of the E1a probe with RNA yielded an extra tract of 835 N which may be identical to the species described above for HindIII-G-transformed cells. The most likely explanation for the aberrant sizes of the E1a-specific mRNAs in HpaI-E-transformed cells is that they lack the viral polyadenylation signal AATAAA which in Ad5 DNA is encoded by residues 1611 to 1616, i.e. just to the right of the HpaI cleavage site at position 1572 (Proudfoot & Brownlee, 1976; Van Ormondt et al., 1980). Consequently, the 3' ends of these RNAs must be of non-viral origin and terminating at a site present in the sequences neighbouring the Ad5 DNA insert. We have no explanation for the fact that E1a-specific RNAs with unusual cap sites only occur in cells belonging to classes II and III. The use of additional E1a promoters has been described for Ad12 both in lytically infected and transformed cells (Sawada & Fujinaga, 1980; Saito et al., 1981).

Of the three E1b-specific RNAs (2.5 kb, 1.15 kb and 0.7 kb) found in lytically infected cells
Fig. 8. Approximate lengths and coordinates of the E1 mRNAs present in cells transformed by the Ad5 fragments XhoI-C, HindIII-G and HpaI-E and at early and late times in lytic infection. The locations are deduced from the results obtained in this study, and from those obtained by Berk & Sharp (1977, 1978), Chow et al. (1979) and Perricaudet et al. (1979, 1980).
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only the early species of 2-5 kb is observed in XhoI-C-transformed cells. Nevertheless, these cells contain two major polypeptides mapping in the E1b subregion (Schrier et al., 1979). Also, the 2-5 kb RNA from infected cells has been shown to direct in vitro the synthesis of both polypeptides (Esche et al., 1980; Lupker et al., 1981). Recently, it was argued on the basis of the nucleotide sequence and detailed nuclease S1 data that the 2-5 kb mRNA indeed codes for both E1b polypeptides which are specified by two partially overlapping open reading frames and initiate at either the 5'-proximal (21 kdal) or the second (55 kdal) AUG triplet (Bos et al., 1981).

The 1-15 kb E1b mRNA, which is only synthesized in lytically infected cells as a late species, also can specify the 21 kdal polypeptide (Bos et al., 1981). Its appearance in large amounts in the late stage probably explains the increase of the 21 kdal protein observed late in infection.

The Ad5 HindIII-G fragment (residues 1 to 2805) contains the following E1b information: the RNA 5'-terminus, the entire coding sequence for the 21 kdal protein, the donor splice site of the late 1-15 kb mRNA, and the code for the N-terminal 262 amino acids of the 55 kdal protein (total length 496 residues), but not the splice sites of the 2-5 kb mRNA and the 3' terminus of the 2-5 kb and 1-15 kb mRNAs. The blotting data show that the E1b-specific RNAs in HindIII-G-transformed cells vary in length between cell lines (Table 1), the shortest species being 1-15 kb long. Annealing of these RNAs to E1b DNA probes yields a single nuclease S1-resistant tract of 1100 N. This value agrees exactly with the distance between their 5' termini, i.e. Ad5 nucleotide 1702 (our unpublished observation), and the HindIII site at position 2805. This implies that the E1b-specific RNAs observed in HindIII-G-transformed cells consist of a 5'-terminal 1100 N-long tract encoded by Ad5 DNA covalently linked to sequences encoded by non-viral DNA, and that the virus-specific portion is not involved in splicing. Our experiments do not answer the question whether the non-viral segment is spliced. The nucleic acid data predict that the HindIII-G-transformed cells contain the 21 kdal E1b polypeptide found in infected cells. This has been confirmed by immunoprecipitation and in vitro protein synthesis studies (Schrier et al., 1979; Jochemsen et al., 1981; P. J. van den Elsen, P. I. Schrier, A. Houweling, B. S. de Pater, J. van der Veer and A. van der Eb, unpublished results).

In conclusion, the data presented in this report show that in cells transformed by DNA fragments only those mRNAs are transcribed from region E1 that are also detected early in the infectious cycle. Transformed cells of classes II and III, which lack the 3' terminus and the polyadenylation signal of the E1b and Ela mRNAs respectively, must contain mRNAs with 3' ends of non-viral origin. These two classes of transformed cells in addition contain an E1a-specific mRNA, hitherto undetected in Ad5-infected or -transformed cells, which initiates at a site upstream from the usual 5' end at nucleotide 499.

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