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Peptide Maps and N-Terminal Sequences of Polypeptides from Early Region 1A of Human Adenovirus 5

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Experiments exploring the reasons for a multiplicity of products from early region 1A of adenovirus 5 are described. Labeled early region 1A products from wild-type virus were synthesized in infected cells and in a cell-free system programmed with mRNA from infected cells, immunoprecipitated specifically with an antipeptide serum, E1A-C1, directed against the C-terminal sequence of E1A products, and separated by gel electrophoresis. Two-dimensional maps of [^{35}S]methionine-labeled peptides were consistent with antigens of 52,000 daltons (52K) and 48.5K being from the 13S mRNA and antigens of 50K, 45K, and 35K from the 12S mRNA. Partial N-terminal sequences of 52K, 50K, 48.5K, and 45K synthesized in vitro showed that each of these antigens was initiated at the predicted ATG at nucleotide 560 in the DNA sequence. These results eliminate multiple initiation sites and proteolytic cleavage at the N-terminal end as sources of antigen diversity. Peptide maps and N-terminal sequences were obtained in a similar way for E1A products from the Ad5 deletion mutant *dl*1504, which lacks the normal initiator codon. As predicted, these polypeptides are initiated at the next ATG, 15 codons downstream in the wild-type sequence. These results are discussed in relation to Kozak's ribosomal scanning model.

In human adenovirus 5 (Ad5) and the closely related human adenovirus 2, genes located in early region 1A (E1A) serve important functions in both the infection and transformation of cells. In the initial stages of infection, a product or products from E1A is responsible for turning on other blocks of early adenovirus genes (2, 18), possibly by interacting with a cellular protein (22) or by facilitating the formation of transcriptional complexes (10). For adenovirus to bring about the transformation of cells, region E1A together with early region 1B are the only portions of the viral genome that are necessary (9, 12).

A puzzling feature of the E1A region of adenovirus is the number of polypeptides produced from it early in infection. Maps of E1A mRNAs (3, 6), the partial sequences of these mRNAs (25), and the complete sequence of the E1A region of the genome (30) suggest that two mRNAs, a 13S of about 1,100 nucleotides and a 12S of about 900 nucleotides, are produced at early times. These mRNAs have the same 5′ and 3′ termini and differ only in the 5′ donor site for the removal of a single intron from the primary transcript. As a result, each mRNA is expected to encode a single polypeptide chain from the same reading frame; the two chains would be 289 and 243 amino acid residues long, they would possess the same N and C terminal ends, and would differ only with respect to an internal stretch of 46 residues. Two early polypeptides have been detected from this region by gel electrophoresis (11, 26), but more frequently the number has been four, six, or more in infected cells treated with drugs and in cell-free translation of selected viral mRNAs (8, 13–15, 17, 28, 29).

Recently, the production of polypeptides from region E1A in cells infected with Ad5 in the complete absence of drugs has been studied by Yee et al. (31). These authors prepared a rabbit antiserum, E1A-C1, against a synthetic heptapeptide corresponding to the C-terminal predicted for products of the 13S and 12S E1A mRNAs and found that it precipitated four major and at least two minor antigens. On sodium dodecyl sulfate (SDS)-polyacrylamide gels, these antigens migrated with M_rs of about 52,000 (52K), 50K, 48.5K, 45K, 37.5K, and 35K. In further studies with this serum on mutants of Ad5, Rowe et al. (27) have shown that the 52K, 48.5K, and 37.5K polypeptides are products of the 13S mRNA, whereas 50K, 45K, and 35K are produced from the 12S mRNA.

More than one polypeptide could be produced from a single mRNA as a result of such factors as multiple translational initiation sites, premature termination, proteolytic cleavage, or post-translational modification. Since the six antigens just described were all immunoprecipitated by the E1A-C1 serum, they must all share the same C-terminal sequence. Thus, none of them could have arisen by premature termination or by cleavage at the C-terminal end (31).

To investigate the products of the E1A region of adenovirus further, particularly with respect to their N-terminal sequences, we obtained tryptic peptide maps and the partial N-terminal sequences of the four major species from wild-type (wt) Ad5. The data show clearly that all four products contain the same N-terminal sequence. As a further check on the expression of region E1A, we obtained peptide maps and partial N-terminal sequences of two major species of polypeptides produced from this region in the adenovirus mutant *dl*1504. This mutant, constructed by Osborne et al. (23), is phenotypically wt but contains a deletion which has removed the AU of the first AUG in the wild-type E1A mRNAs, so that translation is presumed to be initiated at the next AUG, 15 codons further on. This was confirmed.

MATERIALS AND METHODS

Materials. [^{35}S]methionine (specific activity, up to 1,200 Ci/mmol) was from New England Nuclear Corp.; [^{3}H]isoleucine (87 to 113 Ci/mmol) and [^{3}H]leucine (130 to 142 Ci/mmol) were from Amersham Corp. Citrate synthase and oxaloacetate were from Sigma Chemical Co. For amino acid sequencing, reagents were from Beckman Instruments, Inc., and solvents (benzene, ethyl acetate, and butyl chloride) were from Burdick and Jackson Laboratories.

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Viruses. Wt Ad5 was propagated in KB cells and titrated in HeLa cells as previously described (16). The same procedures were also used for the Ad5 deletion mutant *dl*1504 (23), a gift from A. J. Berk to F. L. Graham, McMaster University.

Antisera. Ad5 antigens from early region E1A were immunoprecipitated specifically with a rabbit antiserum (E1A-C1) raised against a synthetic peptide (Tyr-Gly-Lys-Arg-Pro-Arg-Pro) corresponding to the predicted carboxy-terminal ends of the E1A gene products as described by Yee et al. (31). Preimmune sera collected from the rabbits were used as controls.

Synthesis of viral antigens. The methods were essentially those described previously (7). Antigens synthesized in vivo were obtained from whole-cell extracts of KB cells grown in monolayer. The cells were infected at 35 or 50 PFU per cell with wt Ad5 or *dl*1504, labeled with [^{35}S]methionine at 8 to 12 h or 12 to 16 h postinfection, and then harvested. For in vitro synthesis, total cytoplasmic polyadenylated [poly(A)$^+$] mRNA was prepared from KB cells infected with wt Ad5 at 50 PFU per cell or with *dl*1504 at 75 PFU per cell for 8 h in the presence of cycloheximide and translated in a cell-free reticulocyte system. This system was used without further treatment for the translation of mRNA from wt-infected cells, but before translation of mRNA from *dl*1504-infected cells it was depleted of acetyl coenzyme A (CoA) by pretreatment with citrate synthase in the presence of oxaloacetate as a precaution against N-terminal acetylation of nascent proteins (24).

Immunoprecipitation and polyacrylamide gel electrophoresis. Antigens were immunoprecipitated in the presence of protein A-Sepharose beads (7), and the immunoprecipitates were analyzed either by one-dimensional electrophoresis on SDS-polyacrylamide gels (7) or by two-dimensional O'Farrell-type electrophoresis (27), followed in each case by autoradiography.

Peptide mapping and N-terminal sequencing. After one-dimensional gel electrophoresis, polypeptide bands located from the autoradiograph were eluted from the gel and used either for peptide mapping or for sequencing on a Beckman 890C sequencer. (To locate bands of ^{3}H-labeled polypeptides, lanes of ^{35}S-labeled samples were used as markers.) Mapping was performed on oxidized samples digested with trypsin by using thin-layer silica gel plates. Separation was by electrophoresis in 0.33% (vol/vol) pyridine–3.33% (vol/vol) glacial acetic acid in the first dimension followed in the second dimension by chromatography in 35.7% (vol/vol) 1-butanol–28.6% (vol/vol) pyridine–7.1% (vol/vol) glacial acetic acid (5, 7). Sequencing was carried out essentially as described previously (7) on polypeptides that had been identified by peptide mapping part of the sample, or in the case of ^{3}H-labeling, by mapping part of the corresponding ^{35}S-labeled polypeptide used as marker in autoradiographs of the gels.

RESULTS

Comparison of wt E1A products synthesized in vivo and in vitro. KB cells were infected with wt Ad5 or were mock-infected, and at either 8 or 12 h postinfection all of the cells were labeled with [^{35}S]methionine for 4 h. Extracts of these cells were immunoprecipitated with E1A-C1 serum, which is directed against the carboxy terminal sequence predicted for E1A proteins, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. At least six polypeptides were detected in virus-infected cells at both labeling periods (Fig. 1B): four major components with M_rs of 52K, 50K, 48.5K, and 45K, and two minor components of 37.5K and 35K. These species, which closely resemble those reported by Yee et al. (31) with a $^{32}P_i$ label, were not found in uninfected cells (Fig. 1A) or when normal rabbit serum was used instead of E1A-C1 (data not shown).

A pattern similar to that for the four major bands in Fig. 1B was also obtained with E1A products synthesized in vitro (Fig. 1C). A rabbit reticulocyte system was incubated with [^{35}S]methionine in the presence of cytoplasmic poly(A)$^+$ mRNA that had been extracted from KB cells infected with wt Ad5 for 8 h in the presence of cycloheximide. The mixture was immunoprecipitated with either E1A-C1 or normal rabbit serum, and the immunoprecipitates were electrophoresed on an SDS-gel. Four in vitro products were precipitated by E1A-C1 serum (Fig. 1C) but not by normal serum (Fig. 1D). These products were not synthesized in cell-free systems to which had been added either no mRNA or mRNA from uninfected KB cells (data not shown). In vitro, only the two most slowly migrating polypeptides, 52K and 50K, were produced in abundance; the more rapidly migrating species were in much lower relative amounts than they were in vivo (compare Fig. 1C and B). This made it difficult to distinguish in vitro species corresponding to the in vivo 37.5K and 35K products, but in favorable cases bands of 37.5K and 35K were detected with E1A-C1 serum (Fig. 1E). Each of the in vitro polypeptides indicated in Fig. 1E was identified as an E1A product by comparing its peptide map with that of the corresponding in vivo product (see below). (In Fig. 1C, D, and E, the dark uppermost band represents the 72K DNA binding protein from early region 2A, which precipitates nonspecifically from the incubated reticulocyte lysate and which in this gel system migrates at about 60K [J. F. Downey, unpublished data].) For convenience, the E1A products will be referred to by the M_r values shown in Fig. 1A to E, although no special significance can be attached to these values since the products migrate at different rates relative to one another and to molecular weight markers in different gel systems (31; J. F. Downey, unpublished data).

The E1A products synthesized in vivo and in vitro and immunoprecipitated with E1A-C1 antiserum were also compared by two-dimensional electrophoresis, using isoelectric focusing in the first dimension followed by SDS-gel electrophoresis in the second. Figure 1F and G shows the appropriate parts of the autoradiographs in which the four major products can be distinguished. In both cases the pI's of the two sets of products are similar, about 4.8. In Fig. 1G, as in Fig. 1C and E, the intensities of the 48.5K and 45K components are much weaker than those of 52K and 50K, although in Fig. 1G this effect is extreme. In Fig. 1G also, the in vitro products are spread over a wider pH range than those made in vivo. This difference was not observed consistently and is probably due to variations in technique.

The major in vivo and in vitro products were compared further by preparing two-dimensional maps of tryptic peptides of material eluted from gels similar to those in Fig. 1B, C, and E. Autoradiographs of these are shown for the 52K and 48.5K antigens from the 13S mRNA in Fig. 2 and for the 50K, 45K, and 35K antigens from the 12S mRNA in Fig. 3. Although several of the peptides varied in intensity from sample to sample, especially numbers 3 and 4 in 52K (Fig. 2), for example, it was evident that in each case the patterns of [^{35}S]methionine-containing peptides from in vivo and in vitro products were the same. It was also evident from Fig. 2 and 3 and other autoradiographs that the patterns of 52K and 48.5K were identical to one another, as were those for 50K, 45K, and 35K, and that all five antigens share a number of

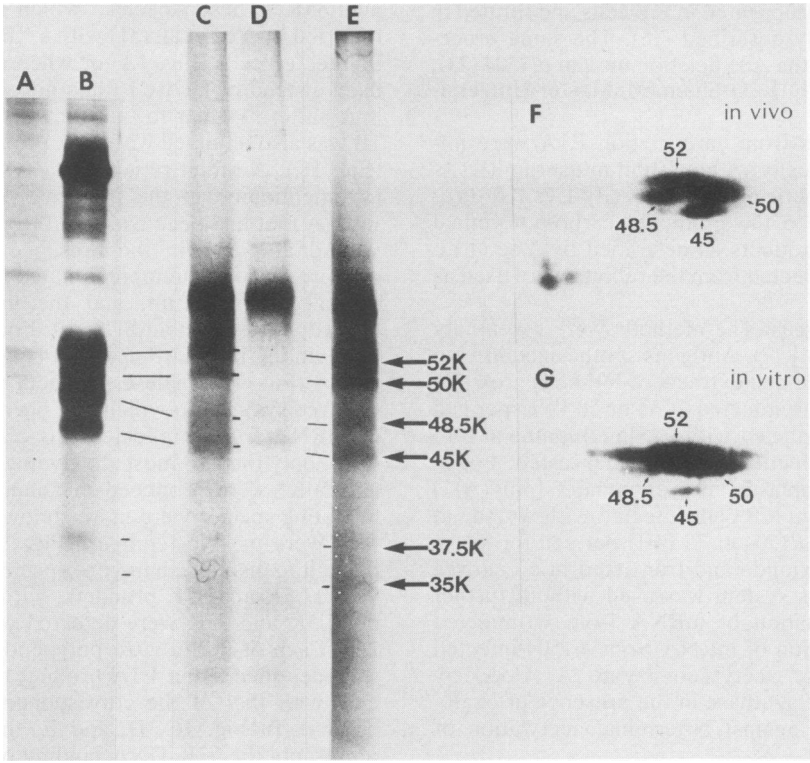

FIG. 1. One- and two-dimensional gel analyses of E1A products synthesized in vivo and in vitro. (A and B) KB cells in monolayer were (A) mock infected or (B) infected with wt Ad5 at 35 PFU per cell and labeled with [^{35}S]methionine (50 μCi per 150-mm dish) from 12 to 16 h postinfection. Extracts of these cells were immunoprecipitated with E1A-C1 serum and separated by electrophoresis on 15% polyacrylamide-SDS gels, and the gels were autoradiographed. In B, the heavy band at the top of the gel is due to hexon, produced in large amount in the labeling period used in this experiment and precipitated nonspecifically. (C, D, and E) Total cytoplasmic poly(A)$^+$ mRNA was isolated from KB cells infected with wt Ad5 for 8 h in the presence of cycloheximide and was translated in a rabbit reticulocyte system containing [^{35}S]methionine. The incubated mixtures were immunoprecipitated with (C and E) E1A-C1 serum or (D) nonimmune serum and analyzed on 12% polyacrylamide-SDS gels, and the gels were autoradiographed. Lanes A through E have been printed to show the minor bands of lower molecular weight; the darker bands were clearly resolved from one another on the original autoradiograph. (F and G) Immunoprecipitates labeled with [^{35}S]methionine were prepared with E1A-C1 serum from (F) infected cells and from (G) a reticulocyte system programmed with mRNA from infected cells, as described above for B and C and E, respectively. These immunoprecipitates were analyzed by two-dimensional electrophoresis, first in pH 4 to 6 isoelectric focusing gels (horizontal dimension) and then in 12% polyacrylamide-SDS slab gels (vertical dimension). Only the portions of the autoradiographs of the gels containing the E1A products are shown. The basic side of the isoelectric focusing dimension is on the left.

peptides. (A similar conclusion was reached by Smart et al. [28] with Ad5 and by Green et al. [13] for the corresponding E1A proteins of adenovirus 2 isolated by two-dimensional electrophoresis.) These results are consistent with 52K and 48.5K being products of the E1A 13S mRNA and 50K, 45K, and 35K being products of the E1A 12S mRNA (27). The DNA sequence predicts that products of the 12S mRNA should differ from those of the 13S in the absence of two ^{35}S-peptides and a change in another ^{35}S-peptide. It is clear that 50K, 45K, and 35K lack peptide 4, which at pH 3.5 migrates towards the anode, but it is not possible to identify another absent peptide or the altered peptide. Apart from peptide 4, the only other consistent difference found in a large number of maps has been the reduction, and in some samples the absence, of peptide 2 from 50K and 45K; this may indicate altered solubility characteristics reflecting a change in the composition of this peptide, even though its position on the map is not detectably different.

Since sequencing was carried out on polypeptides eluted from gels, the purity of such preparations has a bearing on the significance of the sequencing results. In the electrophoretic pattern, the 13S mRNA products alternated with 12S mRNA products, so that the most likely source of contamination was of a product of one mRNA by products of the other. The freedom of 13S mRNA products from contamination by 12S mRNA products cannot be estimated, since the latter contain no peptides not also present in the former. However, the consistent absence of peptide 4 from all of the maps we obtained of 50K, 45K, and 35K shows that these polypeptides were not detectably contaminated with products of the 13S mRNA. This suggests that contamination of any component by its neighbors was probably not significant.

In separate experiments (data not shown), it was found that maps of the 52K and 48.5K products (Fig. 2) were identical to those of the in vivo and in vitro products immunoprecipitated by combined polyvalent hamster antitumor sera, which as Rowe et al. (27) have found precipitate only 13S mRNA products.

Sequencing of the amino terminals of wt in vitro products. To examine the N-termini of the multiple E1A products, syntheses were carried out in the reticulocyte system in the presence of [^{35}S]methionine, [^{3}H]isoleucine, or [^{3}H]leucine. The reaction mixtures were then immunoprecipitated with E1A-C1 serum, the precipitates were electrophoresed on SDS-gels, and material in each of the four main bands was

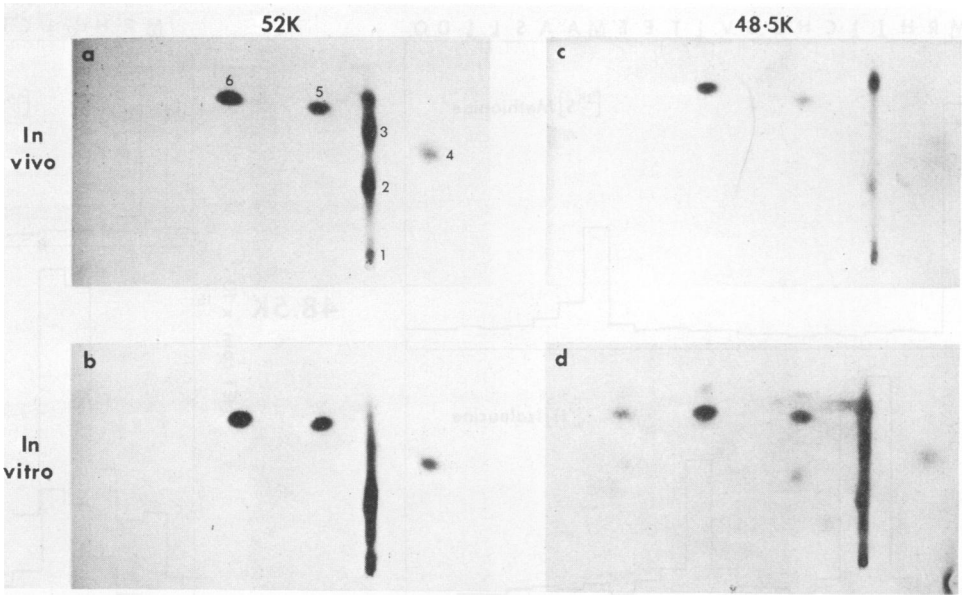

FIG. 2. Maps of [^{35}S]methionine-containing tryptic peptides of (a and b) the 52K and (c and d) the 48.5K products of the E1A 13S mRNA, synthesized (a and c) in vivo and (b and d) in vitro. Polypeptides were eluted from the appropriate bands in gels such as those shown in Fig. 1B and E and digested with tolysulfonyl phenylalanyl chloromethyl ketone-trypsin. Maps were prepared on thin-layer silica gel plates by using electrophoresis in the first (horizontal) dimension and chromatography in the second (vertical) dimension. In each case the origin is toward the lower right (in a, immediately below the spot marked 1). In d some of the fainter spots are due to contaminating peptides.

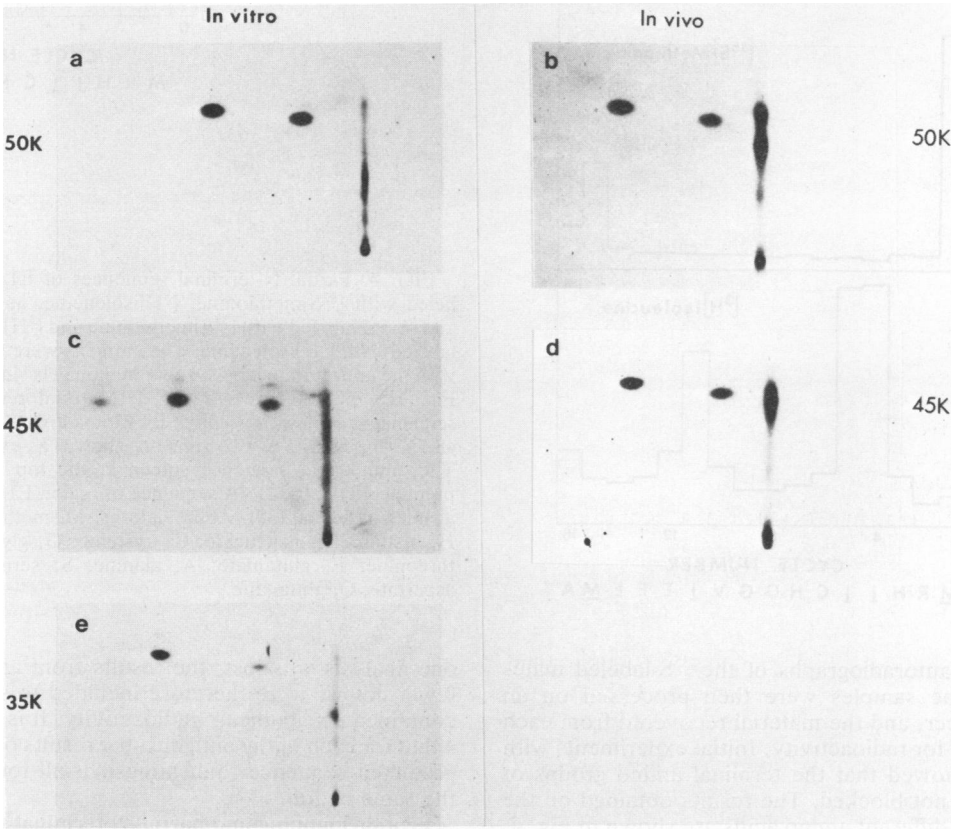

FIG. 3. Maps of [^{35}S]methionine-containing tryptic peptides of (a and b) the 50K, (c and d) 45K, and (e) 35K products of the E1A 12S mRNA, synthesized (a, c, and e) in vitro and (b and d) in vivo. The maps were prepared by procedures similar to those described in the legend to Fig. 2.

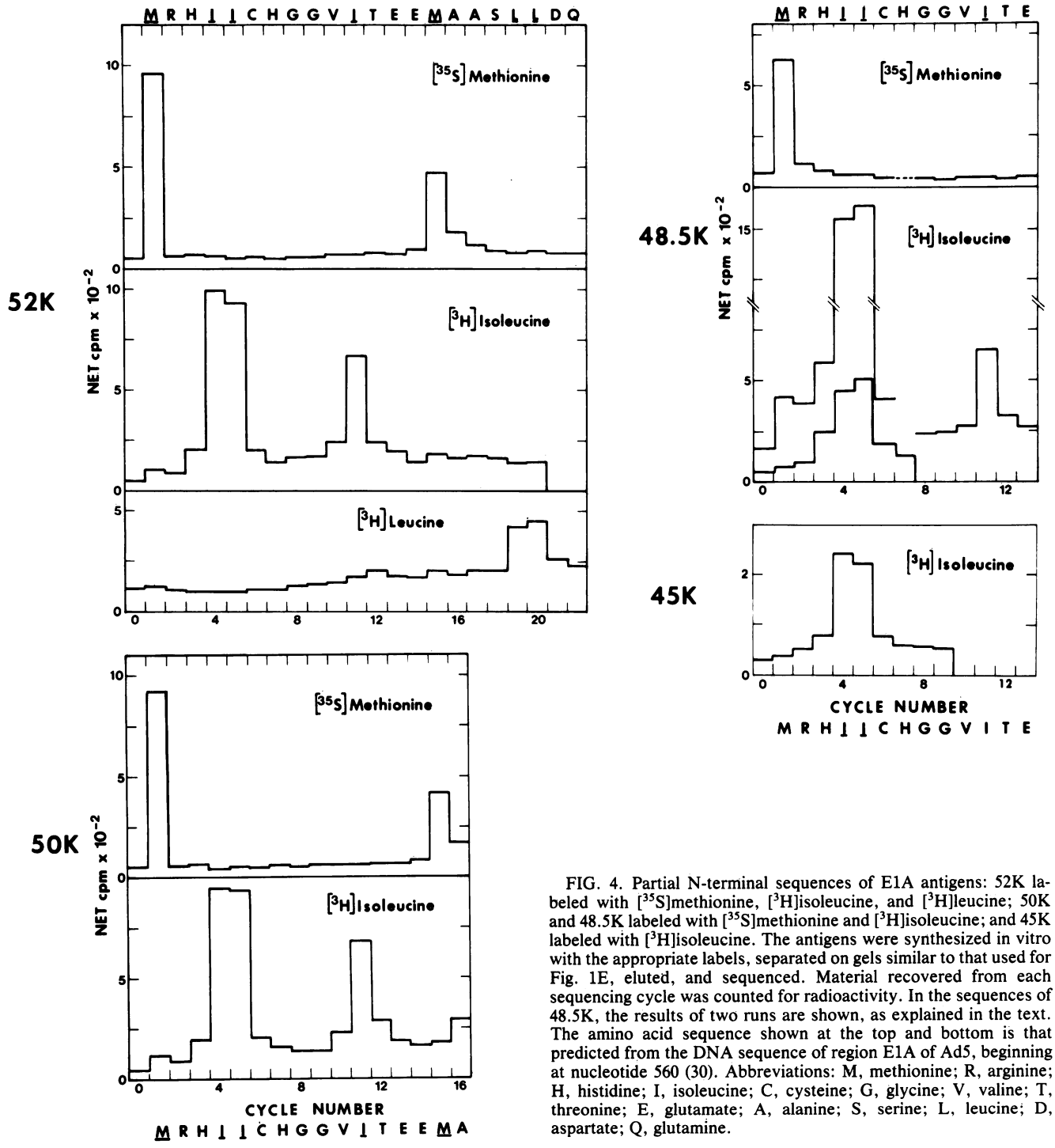

FIG. 4. Partial N-terminal sequences of E1A antigens: 52K labeled with [^{35}S]methionine, [^{3}H]isoleucine, and [^{3}H]leucine; 50K and 48.5K labeled with [^{35}S]methionine and [^{3}H]isoleucine; and 45K labeled with [^{3}H]isoleucine. The antigens were synthesized in vitro with the appropriate labels, separated on gels similar to that used for Fig. 1E, eluted, and sequenced. Material recovered from each sequencing cycle was counted for radioactivity. In the sequences of 48.5K, the results of two runs are shown, as explained in the text. The amino acid sequence shown at the top and bottom is that predicted from the DNA sequence of region E1A of Ad5, beginning at nucleotide 560 (30). Abbreviations: M, methionine; R, arginine; H, histidine; I, isoleucine; C, cysteine; G, glycine; V, valine; T, threonine; E, glutamate; A, alanine; S, serine; L, leucine; D, aspartate; Q, glutamine.

eluted, using the autoradiographs of the ^{35}S-labeled material as guides. The samples were then processed on an automatic sequencer, and the material recovered from each cycle was counted for radioactivity. Initial experiments with [^{35}S]methionine showed that the terminal amino groups of the antigens were not blocked. The results obtained on the four antigens with different amino acids are shown in Fig. 4, together with the sequence predicted by van Ormondt et al. (30) from the DNA sequence for Ad5, beginning at nucleotide 560. For the 48.5K antigen, the sample from cycle 7 in one analysis was lost; the results from an earlier run with fewer counts were therefore included to show that cycle 7 contained no significant radioactivity. It is evident from Fig. 4 that for each of the antigens, the results obtained match the predicted sequence, indicating that all four are initiated at the same codon.

Peptide mapping and partial N-terminal sequencing of E1A products from mutant *dl*1504. E1A products from Ad5 deletion mutant *dl*1504 were synthesized in vivo and in vitro, immunoprecipitated with E1A-C1 serum, and analyzed on

SDS-gel by procedures similar to those for wt virus. Both the in vivo and in vitro products of *dl*1504 gave multiple bands with M_rs roughly comparable to those for wt virus. This is illustrated for the in vitro products of wt and mutant virus in Fig. 5A and B, respectively, where only the most abundant species have been identified. In gels of mutant material synthesized in vitro such as that in Fig. 5B, bands were identified as containing E1A products by eluting the polypeptides and mapping their [^{35}S]methionine peptides. The maps (see Fig. 5C and D) resembled those for wt polypeptides (Fig. 2 and 3) and like the latter formed two groups, namely, those that contained a peptide migrating toward the anode and those that did not. By comparison with the results on wt products, these two groups are assumed to have been synthesized on the 13S and 12S E1A mRNAs respectively.

As explained above, the E1A products of *dl*1504 should be shorter and should therefore migrate faster than those of wt. This was the case for all species except the uppermost band in the in vitro pattern (Fig. 5B). This band, which represents the only noticeable difference between the patterns of in vivo and in vitro products from *dl*1504, was absent in vivo. In the gel shown in Fig. 5B, this band is of roughly the same intensity as the one immediately beneath it. In most experiments, however, it was much weaker so that only the lower of these two bands of 13S product yielded enough material to be sequenced (see below). Whether the presence of the upper band reflects a genuine difference between the synthetic processes in vivo and in vitro or whether it is an artifact of electrophoresis is not known. It is evident that, like the wt E1A products, those from *dl*1504 migrate anomalously on SDS-gels, and so we did not attempt to clarify their electrophoretic patterns further.

If in *dl*1504 E1A products are initiated at the second AUG in the open reading frame, their [^{35}S]methionine peptide maps should lack the dipeptide Met-Arg at the N-terminal of the wt products and should contain an N-terminal peptide of 83 residues in place of a peptide of 95 amino acids (representing residues 3 to 97) in the wt products. It was not possible to identify any spot in the wt maps (Fig. 2 and 3) that was missing from those for *dl*1504 products (Fig. 5C and D) and could represent the dipeptide. The only noticeable difference between the maps is that the spot corresponding to spot 1 in wt (Fig. 2 and 3) chromatographs further in the mutant (Fig. 5), and may therefore represent the large truncated peptide.

To establish the initiation codon for E1A products in *dl*1504, two polypeptides labeled with [^{3}H]leucine were partially sequenced at their N-terminal ends as follows. Material labeled with [^{3}H]leucine was synthesized in a reticulocyte system pretreated with citrate synthase and oxaloacetate as a precaution against N-terminal acetylation. The incubation mixture was immunoprecipitated with E1A-C1 serum, and the immunoprecipitate was electrophoresed on an SDS-gel in parallel with a similar sample labeled with [^{35}S]methionine. With the autoradiograph of the ^{35}S-labeled material as a guide, two bands labeled with [^{35}S]methionine and the corresponding two bands labeled with [^{3}H]leucine

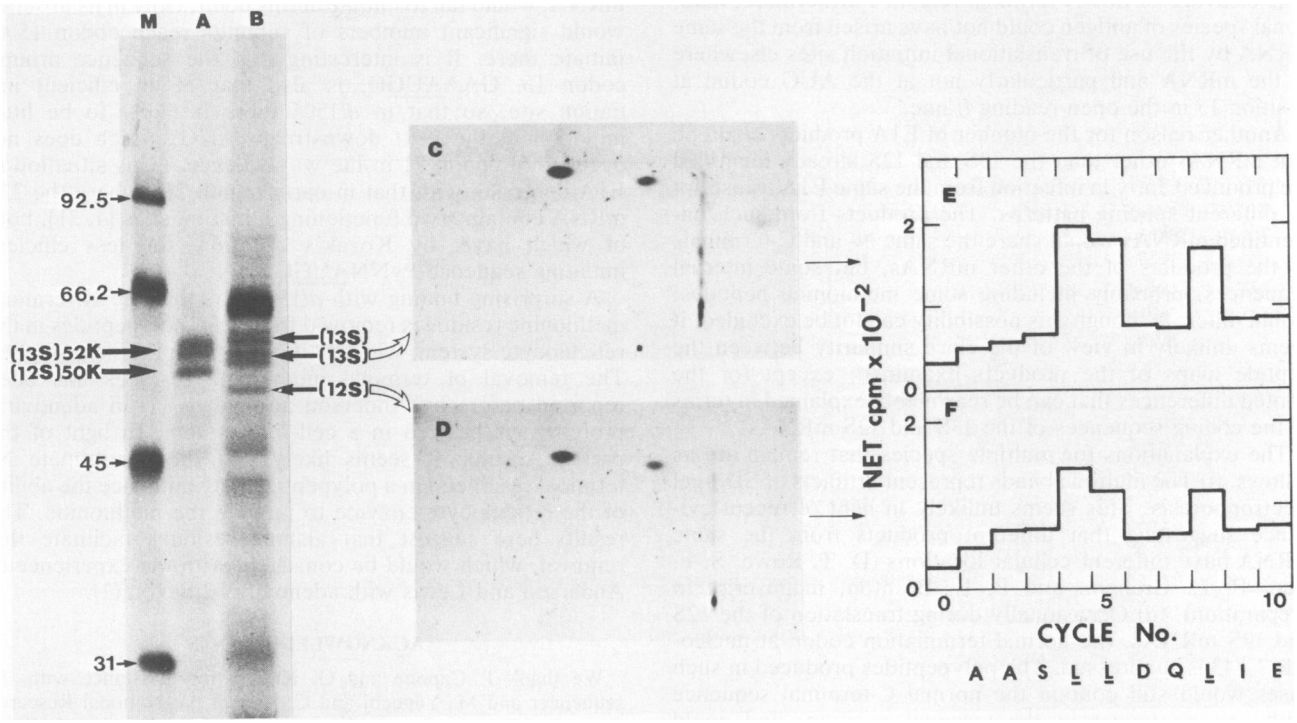

FIG. 5. Gel analyses, peptide maps, and partial N-terminal sequences of E1A products from Ad5 deletion mutant *dl*1504, synthesized in vitro. (M, A, and B) Separation on 12% polyacrylamide-SDS gel of (M) ^{14}C-labeled marker proteins; (A) [^{35}S]methionine-labeled E1A products from wt virus, synthesized in vitro and immunoprecipitated with E1A-C1 serum as described for Fig. 1C and E; and (B) [^{35}S]methionine-labeled E1A products of *dl*1504 prepared by the same procedures as those for A. The electrophoresis conditions were the same as those for Fig. 1A through E. (C and D) Maps of [^{35}S]methionine-containing tryptic peptides of *dl*1504 material eluted from the bands indicated in gels similar to that in B. The numbers in parentheses indicate the E1A mRNA from which the polypeptide in each band is synthesized (see text). The maps were prepared as described in the legend to Fig. 2. (E and F) Partial N-terminal sequences of two *dl*1504 antigens (which originate from 13S and 12S mRNAs, respectively) labeled with [^{3}H]leucine and prepared as described in the legend to Fig. 4. The amino acid sequence under E is that predicted from the DNA sequence for E1A beginning at codon 16. See the legend to Fig. 4 for the abbreviations.

were excised from the gel, and the polypeptides were recovered. The two [^{35}S]polypeptides were used to prepare the peptide maps shown in Fig. 5C and D, whereas the polypeptides labeled with [^{3}H]leucine were partially sequenced (Fig. 5E and F). In both the 13S (Fig. 5E) and the 12S (Fig. 5F) mRNA products, leucine residues were found at the positions predicted by the DNA sequence for a polypeptide initiated at the second AUG codon in the wt reading frame in region E1A but from which the N-terminal methionine has been removed after translation (see the sequence beneath Fig. 5E and F).

The absence of initiating methionine residues from mutant polypeptides recovered from in vitro synthesis was confirmed in a separate experiment in which polypeptides similar to those in Fig. 5B but labeled with [^{35}S]methionine were sequenced. In both cases no radioactivity was recovered in the first two cycles. Analyses on the degradation of lysozyme added to the samples in these experiments showed that the automatic sequencer was performing properly (7).

DISCUSSION

The results presented here eliminate several possible explanations discussed above for the multiplicity of polypeptides from the E1A region of Ad5. First, the partial N-terminal sequences of the four major slowest-migrating species from wt virus, 52K, 50K, 48.5K, and 45K, all correspond to that predicted for E1A products by the DNA sequence starting at nucleotide 560 (30). Thus, none of these antigens could have arisen from one of the others by proteolytic cleavage of this N-terminal region. Furthermore, additional species of antigen could not have arisen from the same mRNA by the use of translational initiation sites elsewhere in the mRNA and particularly not at the AUG codon at position 15 in the open reading frame.

Another reason for the number of E1A products could be that mRNAs other than the 13S and 12S already identified are produced early in infection from the same E1A transcript by different splicing patterns. The products from such unidentified mRNAs would share the same N- and C-terminals as the products of the other mRNAs, but some internal sequences, probably including some methionine peptides, would differ. Although this possibility cannot be excluded, it seems unlikely in view of the close similarity between the peptide maps of the products examined, except for the limited differences that can be reasonably explained in terms of the coding sequences of the 13S and 12S mRNAs.

The explanations for multiple species that remain are as follows. (i) The multiple bands represent artifacts of SDS-gel electrophoresis. This seems unlikely in light of recent evidence suggesting that different products from the same mRNA have different cellular locations (D. T. Rowe, S.-P. Yee, F. L. Graham, and P. E. Branton, manuscript in preparation). (ii) Occasionally during translation of the 12S and 13S mRNAs, the normal termination codon at nucleotide 1,543 is suppressed. The polypeptides produced in such cases would still contain the normal C-terminal sequence (although no longer in the terminal position) and could therefore still respond to the E1A-C1 serum. Since no methionine occurs in the normal C-terminal tryptic peptide or among the residues that would be added, the peptide maps would not detect suppression of termination. An argument against this possibility comes from results with Ad5 deletion mutant *dl*313. In this mutant, the 3′ end of region E1A and most of early region 1B are removed, so that translation initiated in E1A is terminated by sequences at the extreme 3′ end of early region 1B. Despite the lack of the normal E1A termination sequence, in infected cells this mutant still produces at least two polypeptides from the mutated E1A 13S mRNA (27). To explain this result, suppression of termination would have to be widespread in the adenoviral genome. This does not seem likely as it would have serious consequences for growth of the virus. (iii) There are different degrees of post-translational modification of the product molecules. This seems to be the most likely explanation, although at present the nature of the modifications responsible is unclear. Yee et al. (31) have shown that all the E1A polypeptides are phosphorylated, and there was an indication from ^{32}P-peptide maps that the antigens may differ in the degree of phosphorylation. Whether these differences give rise to the multiple species remains to be determined.

The N-terminal sequences obtained for the E1A products of mutant *dl*1504 confirmed that the initiation of translation of these products occurs at the codon predicted, namely, codon 15 in the open reading frame of the wt sequence. Furthermore, these sequences and those for the wt polypeptides establish clearly that translation can be initiated at the AUG codon at position 15 but that this codon is not used as an initiator until the normal initiator at codon position 1 has been deleted. This confirms what would be predicted by Kozak's modified scanning model for initiation of translation in eucaryotic mRNAs (19, 20). The sequence around codon 1, AAAAUGA, conforms to the consensus sequence PuNNAUGPu that Kozak has identified for efficient initiation sites. In the presence of this site, therefore, very few 40S ribosomal subunits, as they scan from the 5′ end of the mRNA, would fail to initiate at this point. Only in its absence would significant numbers of subunits reach codon 15 to initiate there. It is interesting that the sequence around codon 15, GAAAUGG, is also that of an efficient initiation site, so that in *dl*1504 there is likely to be little initiation at the next downstream AUG, which does not occur until codon 71 in the wt sequence. (This situation in E1A contrasts with that in early region 1B, where the 22S mRNA contains two functioning initiation sites [4, 21], both of which have, by Kozak's criterion, the less efficient initiating sequence PyNNAUGG.)

A surprising finding with *dl*1504 was that the N-terminal methionine residue is removed from E1A polypeptides in the reticulocyte system, whereas it is not from the wt products. The removal of terminal methionine residues has been reported before by Anderson and Lewis (1) in adenoviral proteins synthesized in a cell-free system. In light of the present results, it seems likely that the penultimate N-terminal residue(s) in a polypeptide may influence the ability of the reticulocyte enzyme to remove the methionine. The results here suggest that alanine residues facilitate this removal, which would be consistent with the experience of Anderson and Lewis with adenovirus 2 hexon (1).

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LITERATURE CITED

1. **Anderson, C. W., and J. B. Lewis.** 1980. Amino-terminal sequence of adenovirus type 2 proteins: hexon, fiber, component

IX, and early protein 1B-15K. Virology **104**:27–41.
2. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. A. Sharp.** 1979. Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
3. **Berk, A. J., and P. A. Sharp.** 1978. Structure of the adenovirus 2 early mRNAs. Cell **14**:695–711.
4. **Bos, J. L., L. J. Polder, R. Bernards, P. I. Schrier, P. J. van den Elsen, A. J. van der Eb, and H. van Ormondt.** 1981. The 2.2Kb E1B mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. Cell **27**:121–131.
5. **Branton, P. E., N. J. Lassam, J. F. Downey, S.-P. Yee, F. L. Graham, S. Mak, and S. T. Bayley.** 1981. Protein kinase activity immunoprecipitated from adenovirus-infected cells by sera from tumor-bearing hamsters. J. Virol. **37**:601–608.
6. **Chow, L. T., T. R. Broker, and J. B. Lewis.** 1979. Complex splicing patterns of RNAs from the early regions of adenovirus 2. J. Mol. Biol. **134**:265–303.
7. **Downey, J. F., D. T. Rowe, S. Bacchetti, F. L. Graham, and S. T. Bayley.** 1983. Mapping of a 14,000-dalton antigen to early region 4 of the human adenovirus 5 genome. J. Virol. **45**:514–523.
8. **Esche, H., M. B. Mathews, and J. B. Lewis.** 1980. Proteins and messenger RNAs of the transforming region of wild-type and mutant adenoviruses. J. Mol. Biol. **142**:399–417.
9. **Gallimore, P. H., P. A. Sharp, and J. Sambrook.** 1974. Viral DNA in transformed cells. II. A study of the sequences of Ad2 DNA in nine lines of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol. **89**:49–72.
10. **Gaynor, R. B., and A. J. Berk.** 1983. Cis-acting induction of adenovirus transcription. Cell **33**:683–693.
11. **Gaynor, R. B., A. Tsukamoto, C. Montell, and A. J. Berk.** 1982. Enhanced expression of adenovirus transforming proteins. J. Virol. **44**:276–285.
12. **Graham, F. L., A. J. van der Eb, and H. L. Heijneker.** 1974. Size and location of the transforming region in human adenovirus DNA. Nature (London) **251**:687–691.
13. **Green, M., W. S. M. Wold, K. H. Brackmann, and M. A. Cartas.** 1979. Identification of families of overlapping polypeptides coded by early "transforming" gene region 1 of human adenovirus type 2. Virology **97**:275–286.
14. **Halbert, D. N., and H. J. Raskas.** 1982. Tryptic and chymotryptic methionine peptide analysis of the in vitro translation products specified by the transforming region of adenovirus type 2. Virology **116**:406–418.
15. **Halbert, D. N., D. J. Spector, and H. J. Raskas.** 1979. In vitro translation products specified by the transforming region of adenovirus type 2. J. Virol. **31**:621–629.
16. **Harrison, T., F. Graham, and J. Williams.** 1977. Host-range mutants of adenovirus type 5 defective for growth in HeLa cells. Virology **77**:319–329.
17. **Harter, M. L., and J. B. Lewis.** 1978. Adenovirus type 2 early proteins synthesized in vitro and in vivo: identification in infected cells of the 38,000- to 50,000-molecular-weight protein encoded by the left end of the adenovirus type 2 genome. J. Virol. **26**:736–749.
18. **Jones, N., and T. Shenk.** 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. U.S.A. **76**:3665–3669.
19. **Kozak, M.** 1981. Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res. **9**:5233–5252.
20. **Kozak, M.** 1983. Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiol. Rev. **47**:1–45.
21. **Lupker, J. H., A. Davis, H. Jochemsen, and A. J. van der Eb.** 1981. In vitro synthesis of adenovirus type 5 antigens. I. Translation of early region 1-specific RNA from lytically infected cells. J. Virol. **37**:524–529.
22. **Nevins, J. R.** 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell **26**:213–220.
23. **Osborne, T. F., R. B. Gaynor, and A. J. Berk.** 1982. The TATA homology and the mRNA 5′ untranslated sequence are not required for expression of essential adenovirus E1A functions. Cell **29**:139–148.
24. **Paucha, E., A. Mellor, R. Harvey, A. E. Smith, R. M. Hewick, and M. D. Waterfield.** 1978. Large and small tumor antigens from simian virus 40 have identical amino termini at 0.65 map units. Proc. Natl. Acad. Sci. U.S.A. **75**:2165–2169.
25. **Perricaudet, M., G. Akusjärvi, A. Virtanen, and U. Pettersson.** 1979. Structure of two spliced mRNAs from the transforming region of human subgroup C adenoviruses. Nature (London) **281**:694–696.
26. **Ricciardi, R. P., R. L. Jones, C. L. Cepko, P. A. Sharp, and B. E. Roberts.** 1981. Expression of early adenovirus genes requires a viral encoded acidic polypeptide. Proc. Natl. Acad. Sci. U.S.A. **78**:6121–6125.
27. **Rowe, D. T., S.-P. Yee, J. Otis, F. L. Graham, and P. E. Branton.** 1983. Characterization of human adenovirus type 5 early region 1A polypeptides using antitumor sera and an antiserum specific for the carboxy terminus. Virology **127**:253–271.
28. **Smart, J. E., J. B. Lewis, M. B. Mathews, M. L. Harter, and C. W. Anderson.** 1981. Adenovirus type 2 early proteins: assignment of the early region 1A proteins synthesized *in vivo* and *in vitro* to specific mRNAs. Virology **112**:703–713.
29. **van der Eb, A. J., H. van Ormondt, P. I. Schrier, J. H. Lupker, H. Jochemsen, P. J. van den Elsen, R. J. DeLeys, J. Maat, C. P. van Beveren, R. Dijkema, and A. de Waard.** 1979. Structure and function of the transforming genes of human adenoviruses and SV40. Cold Spring Harbor Symp. Quant. Biol. **44**:383–399.
30. **van Ormondt, H., J. Maat, and C. P. van Beveren.** 1980. The nucleotide sequence of the transforming early region E1 of adenovirus type 5 DNA. Gene **11**:299–309.
31. **Yee, S.-P., D. T. Rowe, M. L. Tremblay, M. McDermott, and P. E. Branton.** 1983. Identification of human adenovirus early region 1 products by using antisera against synthetic peptides corresponding to the predicted carboxy termini. J. Virol. **46**:1003–1013.