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Mapping of a 14,000-Dalton Antigen to Early Region 4 of the Human Adenovirus 5 Genome

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An early 14,000-dalton (14K) antigen of adenovirus 5, hitherto designated 10.5K and thought to be from early region 1 (E1), has been shown to be a product of region E4 on the following evidence. In KB cells infected with the adenovirus 5 mutants *dl*312 and *dl*313, containing large deletions in region E1, this antigen was produced in a form having the same mobility as that in wild-type infections. In a range of rodent cells transformed by adenovirus 5 DNA, the synthesis of 14K antigen and the ability of these cells to elicit an immune response to this protein both correlated with the presence of sequences from region E4 of the viral genome. A 14K polypeptide was synthesized in a cell-free system programmed with infected-cell mRNA and was found to be identical to the in vivo antigen in antigenicity, in electrophoretic mobility, and in [^{35}S]methionine-containing tryptic peptides. After labeling with [^{35}S]methionine and several ^{3}H-amino acids, this in vitro product gave an N-terminal sequence identical to that expected from one of the open reading frames (reading region 3) in the DNA sequence for region E4 of Hérissé et al. (Nucleic Acids Res. **9**:4023–4042, 1981). It is likely that this antigen is the same as the nucleus-associated 11K polypeptide from E4 described by other authors.

A number of recent studies on human adenovirus types 2 and 5 (Ad2 and Ad5) (e.g., 3, 8) have been concerned with identifying the polypeptides encoded by early region 1 (E1) of the viral genome, the portion of the viral DNA known to be responsible for cell transformation. The task of establishing what these polypeptides are has been complicated by the low levels at which they are synthesized in infected cells relative to the levels of host proteins and by the difficulty of correlating polypeptides with mRNAs identified from this region. In a previous paper (21), we reported a 10,500-dalton (10.5K) polypeptide in cells infected with wild-type (*wt*) Ad5 that was produced in much reduced amounts by host range (*hr*) Ad5 mutants of group 1 mapping in region E1A. The reduced synthesis with group 1 mutants suggested that this polypeptide may have been encoded in region E1A, but we were unable to establish this assignment by immunological studies as the results with appropriate sera were not clear-cut. Subsequently, Ross et al. (27, 28) assigned a similar, 10K polypeptide to region E1A on the basis that it was immunoprecipitated by antitumor sera specific for antigens from region E1 and that it was not produced by host range deletion mutants of Ad5 mapping in E1A. A difficulty with accepting the assignment of a polypeptide of this size to region E1A was that there was no evidence for an mRNA from this region which could encode it. We therefore examined this question further, and we show here, from studies on deletion mutants of Ad5, on cells transformed with restriction fragments of Ad5 DNA, and by amino acid sequencing, that the 10.5K polypeptide we reported earlier (21), and which is more accurately described as 14K, is encoded by early region E4 at the right-hand end of the viral genome.

MATERIALS AND METHODS

Materials. [^{35}S]methionine (specific activity, up to 1,200 Ci/mmol) was from New England Nuclear Corp.; [^{3}H]alanine (36 Ci/mmol), [^{3}H]isoleucine (87 Ci/mmol), and [^{3}H]leucine (147 Ci/mmol) were from Amersham Corp.; and [α-^{32}P]dCTP (up to 800 Ci/mmol) was from New England Nuclear. For amino acid sequencing, reagents were from Beckman Instruments, Inc., and solvents (benzene, ethyl acetate, and butyl chloride) were from Burdick and Jackson Laboratories.

Viruses. Ad5 *wt* was propagated in KB cells and titrated in HeLa cells as previously described (10, 12). The Ad5 deletion mutants *dl*312 and *dl*313 (gifts from T. Shenk) and host range mutants (12) were propagated and titrated on 293 cells (10, 11).

Transformed cells. A number of different Ad5-transformed cell lines were used in this work (see Table 1).

Line 945-4 and its subclone 945-C1 and line 954-21 and its subclone 954-C4 were established from primary baby hamster kidney cells transformed by a total *Hin*dIII digest of *wt* Ad5 DNA. Line 945-C1T was established from a tumor induced by 945-C1. Line 954-5 is a baby hamster kidney cell line transformed by purified *Hin*dIII fragment G (left 8%) of *wt* Ad5 DNA, and 972-2, 972-3, 983-2, and 983-3 were transformed by purified *Xho*I-C (left 16%). Line 1019-1 and subclones 1019-1SC1 and 1019-C1 and line 1019-2 were transformed by a total *Xho*I digest of *hr*6 DNA, whereas 1019-3 was transformed by an *Xho*I digest of *hr*50. Line 1019-1T was established from a tumor induced by 1019-1. A detailed characterization of these lines will be published elsewhere (Rowe and Graham, manuscript in preparation). The 268-C3 cells are a baby hamster kidney line transformed by sheared *wt* Ad5 DNA and have been described elsewhere (35). Likewise, 424-C1, a rat cell line transformed by sonicated Ad5 DNA, and 512-C8, a rat cell line transformed by purified *Hin*dIII fragment G, have been described previously (21, 35).

All of the above transformed rodent cells were cultured in Joklik modified minimal essential medium plus 10% newborn calf serum.

Southern blot analysis. DNA was extracted from transformed cells as previously described (29), digested with restriction endonuclease *Hin*dIII (Bethesda Research Laboratories), and electrophoresed through 1% agarose gels. The DNA was then transferred to nitrocellulose filters by the method of Southern (31) as modified by Wahl et al. (36). For hybridization, DNA from plasmids containing *Hin*dIII fragment I (97 to 100 map units), F (89 to 97), G (0 to 8), or E (8 to 17) of Ad5 DNA was labeled in vitro with [α-^{32}P]CTP by nick translation according to Maniatis et al. (25). Hybridization was performed at 42°C in 50% formamide as described by Wahl et al. (36).

Infection, labeling, and extraction of cells. About 10^7 KB cells in monolayer in a 150-mm dish were infected with 50 PFU of *wt* or *dl* mutant Ad5 per cell in 1.0 ml of α-minimal essential medium (32). Ninety minutes later, α-minimal essential medium supplemented with 2% fetal calf serum or 5% horse serum was added. To label the cells, the medium was removed, the cells were rinsed briefly with a small volume of methionine-free medium, and then 4 ml of methionine-free medium containing 50 or 100 μCi of [^{35}S]methionine was added. After labeling for 2 or 4 h, the cells were harvested by scraping and washed three times in Tris-saline buffer (0.137 M NaCl, 5 mM KCl, 0.1 mM Na_2HPO_4, 5.5 mM D-glucose, 25 mM Tris, pH 7.4) with centrifuging.

Whole-cell extracts were prepared by suspending cells from one 150-mm dish in 1 ml of RIPA buffer (1% [wt/vol] sodium deoxycholate, 1% [vol/vol] Triton X-100, 0.2% [wt/vol] sodium dodecyl sulfate [SDS], 150 mM NaCl, 50 mM Tris, pH 7.4), freezing and thawing once, sonicating (Biosonic III, setting 30) to disperse aggregated material, and then pelleting debris at 6,500 $\times$ *g* for 1 min.

Cell-free protein synthesis. Total cytoplasmic polyadenylated [poly(A)$^+$] mRNA was prepared from KB cells grown to a density of about 4×10^5/ml in suspension culture in Joklik modified minimal essential medium supplemented with 5% horse serum and infected at a multiplicity of 50 PFU per cell, or mock-infected, for 8 h in the presence of 25 μg of cycloheximide (Sigma Chemical Co.) per ml (14). Cells were harvested and washed twice in Tris-saline buffer, and poly(A)$^+$ mRNA was prepared from the cytoplasm by the method of Wheeler et al. (38). The mRNA was translated either in a nuclease-treated, cell-free rabbit reticulocyte system prepared after the method of A. E. Smith (personal communication) or in a Krebs II mouse ascites system (34). Every translation reaction contained 19 unlabeled and 1 radioactive amino acid at 1.33 mCi/ml for [^{35}S]methionine and at 0.5 mCi/ml for each of the ^{3}H-labeled amino acids. The [^{35}S]methionine was added directly; the ^{3}H-labeled amino acids were freeze-dried and redissolved in water before use.

Antisera and immunoprecipitation. Ad5 antigens were immunoprecipitated from labeled cell extracts and from incubations for cell-free protein synthesis, using antitumor sera from hamsters bearing tumors induced by different lines of Ad5-transformed cells. Serum batches were usually obtained by pooling sera collected from 10 to 20 animals. Of the lines used, 14b cells contain the left 40% of the Ad5 genome and transcribe region E1 (9); the other lines are described above. In addition to these individual sera, a polyvalent serum made by pooling several different sera (D. T. Rowe, P. E. Branton, and F. L. Graham, manuscript in preparation) was also used.

To each milliliter of whole-cell extract were added 3 μl of antiserum and 250 μl of a 10% (vol/vol) suspension of equilibrated protein A-Sepharose beads (Pharmacia Fine Chemicals, Inc.) in RIPA buffer. To each 30-μl (reticulocyte) or 25-μl (ascites) cell-free incubation were added 1 ml of RIPA buffer, 2.5 μl of serum, and 200 μl of the 10% bead suspension. In each case, the mixture was then shaken continuously at 4°C for 2 h or more, after which the beads were pelleted at 350 $\times$ *g* for 1 min, washed three times with fresh buffer, suspended in 50 μl of electrophoresis sample buffer (0.625 M Tris, pH 6.8, 2% [wt/vol] SDS, 0.1 M dithiothreitol, 10% [vol/vol] glycerol, 0.02% bromophenol blue), and incubated on ice for 20 min. Before electrophoresis, the suspensions of beads were boiled for at least 2 min.

SDS-polyacrylamide gel electrophoresis and autoradiography. The procedures for SDS-polyacrylamide gel electrophoresis and autoradiography were essentially as described previously (21). Electrophoresis was carried out in 15% acrylamide gels. When polypeptides were to be recovered, gels were dried under vacuum without further treatment. Other gels were stained with 0.1% Coomassie brilliant blue, destained, and then dried. Autoradiographs were made on Kodak X-Omat film, types XRP-1, XR-5, and XAR-5.

Elution of polypeptides from polyacrylamide gels. The method for elution of polypeptides was similar to that of Collett et al. (6). The band to be eluted was located from the autoradiograph for ^{35}S-labeled polypeptides or, in the case of ^{3}H-labeled material, in relation to the autoradiographs of marker lanes of similar ^{35}S-labeled samples. The appropriate strip of gel was excised, diced, rehydrated in 0.05 M ammonium bicarbonate–3.5 mM SDS (pH 8.5), and incubated at 41°C for 16 h. Debris was removed by centrifugation, and to the supernatant were added carrier protein (100 μg of bovine serum albumin for tryptic peptide mapping or 300 μg of lysozyme for sequencing) and then trichloroacetic acid to 20% (wt/vol). The precip-

itated protein was washed once with 20% trichloroacetic acid.

Tryptic peptide mapping. The washed, precipitated protein was oxidized in performic acid at 4°C for 2.5 h (16), diluted with water, freeze-dried, redissolved in water, and freeze-dried again. It was then redissolved in 50 mM ammonium bicarbonate, pH 8.5, and digested three times with 20 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington Diagnostics), each for 4 h or more at 37°C. Digested samples were freeze-dried, dissolved in water, freeze-dried again, and finally dissolved in a small volume of water. Peptide maps were prepared on thin-layer silica gel plates (20 by 20 by 0.02 cm; Polygram SIL NH-R; Macherey-Nagel Co.), two samples to a plate. Electrophoresis at 450 V in 0.33% (vol/vol) pyridine–3.33% (vol/vol) glacial acetic acid was 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. After drying, the plates were either impregnated with 7% (wt/vol), 2,5-diphenyloxazole in acetone or dipped in molten naphthalene containing 0.4% (wt/wt) 2,5-diphenyloxazole, dried, and autoradiographed.

Sequencing of the amino terminal of 14K antigen. Sequencing was carried out on a Beckman 890C sequencer with a modified cold trap, using the Beckman 0.1 M Quadrol buffer program with slight modifications. Before a sample of antigen was sequenced, 4 mg of Polybrene and 100 nmol of glycylglycine, both in aqueous solution, were applied to the spinning cup of the sequencer, and three cycles of sequencing were performed. This was to remove any amino-blocking compounds in the Polybrene with the glycylglycine. The antigen, after elution from the gel and precipitation and washing with trichloroacetic acid, was dissolved in 50% (vol/vol) formic acid. Some ^{35}S-labeled samples were sequenced on their own, but ^{3}H-labeled samples were mixed with a ^{35}S-labeled sample for sequencing. This was because the release of ^{35}S counts from the N-terminal methionine was a convenient, immediate indication that sequencing of the labeled sample had begun. The antigen sample was applied to the cup, together with 3 to 4 mg of salt-free lysozyme (Worthington) to serve as an internal control. Before sequencing, a zero run was carried out without addition of phenylisothiocyanate, so as to remove any radioactive material soluble in ethyl acetate or butyl chloride. The material recovered from each cycle of sequencing was dried at 70°C, resuspended in methanol, mixed with 20 volumes of Aquasol, and counted for radioactivity in a liquid scintillation counter. In samples containing ^{3}H and ^{35}S, the total net counts per minute for each isotope were calculated by standard procedures from counts recorded in separate energy channels. The performance of the sequencer was monitored at intervals by checking the degradation of the lysozyme. In cycles in which this was done, only 75% of the recovered material was counted. The remainder was dried, and the anilinothiazolinone residues were converted to their phenylthiohydantoin derivatives. They were then identified by comparison with standards by chromatography on Polygram silica thin-layer plates in 88.9% (vol/vol) xylene–11.1% (vol/vol) methanol (17). The counts per minute per radioactive residue were in the range of 25 to 58% of the theoretical yield. The repetitive yields of radioactive amino acids and of unlabeled amino acids from lysozyme were both regularly 93 to 96%. In retrospect, we found that to obtain significant results it was usually necessary to load at least 2,000 cpm per labeled residue in the sequence being analyzed.

RESULTS

Production of 14K antigen in cells infected with Ad5 deletion mutants *dl*312 and *dl*313. To be able to study the 14K antigen, we required an antitumor serum capable of immunoprecipitating it efficiently. In our experience (Rowe and Graham, unpublished data), different Ad5-transformed cell lines raise antibodies against different antigens, the response obtained generally being characteristic of the cell line injected. Therefore, to obtain antisera against a wide variety of tumor antigens, it is necessary to raise sera against a large number of cell lines. Recently, we established a hamster line, 945-C1, transformed by a *Hin*dIII digest of Ad5 DNA and found that hamsters bearing tumors induced by these cells gave a strong immune response to 14K antigen, the resulting antitumor serum being nearly monospecific for this polypeptide (Fig. 1). A related cell line, 945-4 (see Materials and Methods), induced the same strong response to 14K as did 945-C1. By contrast, 14b antitumor serum was incapable of precipitating the 14K antigen from whole-cell extracts (Fig. 1). In a number of experiments to be described, 945-C1 serum was pooled with several different antitumor sera having greater avidities for other viral antigens (Rowe et al., in preparation) to produce a polyvalent serum that could immunoprecipitate 58K and 19K early antigens (believed to be from region E1B) and a group of early antigens of about 44K (probably from E1A), in addition to the 14K (Fig. 2).

To ascertain whether the coding sequences for 14K lay within E1, we examined the ability of Ad5 mutants *dl*312 and *dl*313 (18) with deletions in E1 to synthesize this polypeptide. The deletion in *dl*312 extends from nucleotide 448 to 1,349 (28) and includes the promoter and most of the coding sequences of E1A (nucleotides 468 to 1,632) (33). In *dl*313, on the other hand, a large deletion from nucleotide 1,334 to 3,639 inclusive (5) has resulted in the fusion of the 5′ end of E1A to the 3′ end of E1B. Thus, if 14K were encoded within E1, one or both of these deletion mutants should be unable to synthesize it in its wild-type form.

Analysis of viral antigens immunoprecipitated from KB cells infected with *wt* Ad5 or with one of the two deletion mutants are shown in Fig. 2. Ad5 *wt*-infected cells contained 58K, 44K, 19K, and 14K antigens. In cells infected with *dl*312, reduced amounts of 58K, 19K, and 14K were seen, but no 44K could be detected. In infections with *dl*313, only the 14K antigen was

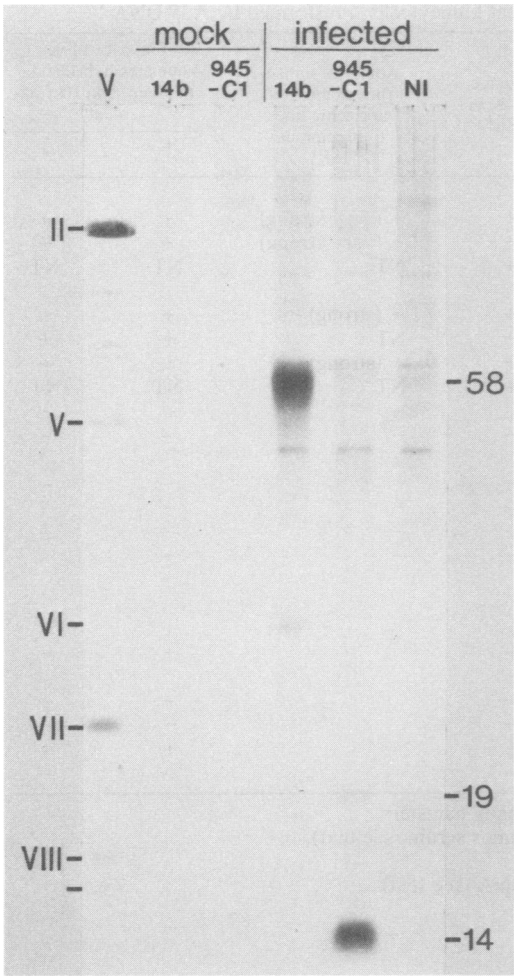

FIG. 1. Analyses of early Ad5 antigens immunoprecipitated by different antitumor sera. Mock-infected KB cells or KB cells infected with *wt* Ad5 for 9 h were labeled with [^{35}S]methionine 7 to 9 h postinfection. Whole-cell extracts of these cells were immunoprecipitated with sera raised against tumors induced by 14b or by 945-C1 cells, or with nonimmune hamster serum, and the precipitates were analyzed on SDS-gels, which were then autoradiographed as described in the text. V, marker lane of [^{35}S]methionine-labeled Ad5 virion proteins.

detected, again in an amount much lower than with *wt* infections.

These results are consistent with the assignment of the antigens of molecular weights of about 44,000 to region E1A and those of 58,000 and 19,000 molecular weights to E1B. However, that the two deletion mutants both produced a 14K antigen of identical mobility to that of the *wt* product, and not in a truncated form or enlarged by fusion to another polypeptide, indicated that the 14K antigen could not be encoded by E1. The reduced levels of 14K synthesis with the deletion mutants can be explained in light of previous results with E1A *hr* mutants, which indicate that an E1A product is required for the full expression of other early regions of the genome (1, 19).

It seemed likely, therefore, that the 14K anti-

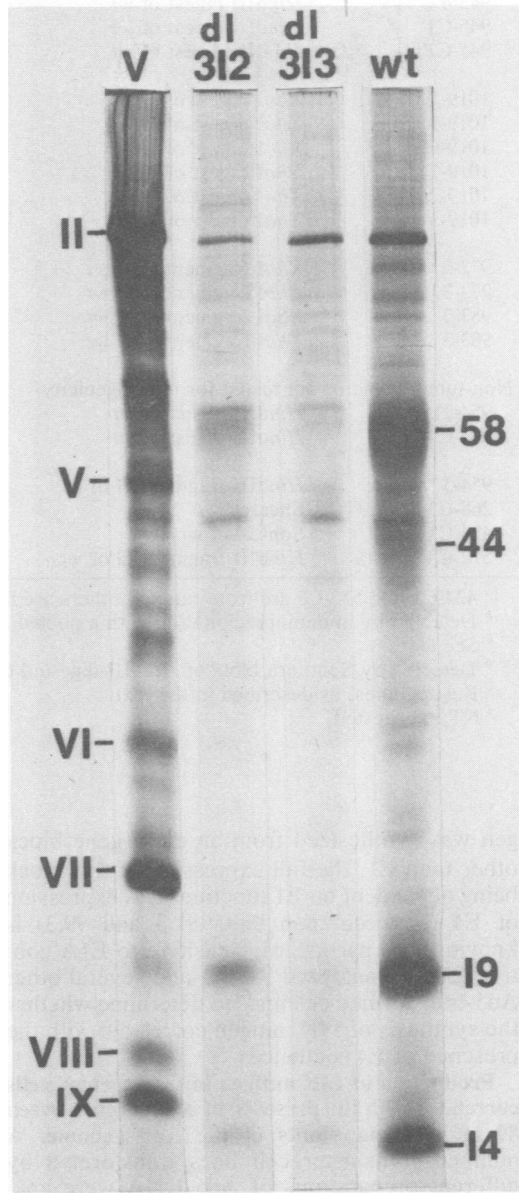

FIG. 2. Early Ad5 antigens expressed in KB cells infected with *wt* or deletion mutant virus. Cells were infected for 9 h at a multiplicity of 50 PFU per cell with *wt*, mutant *dl*312, or mutant *dl*313 virus and labeled 7 to 9 h postinfection with [^{35}S]methionine. Whole-cell extracts were immunoprecipitated with a pooled polyvalent antitumor serum (see text), and the precipitates were analyzed on SDS-gels. V, Virus marker.

TABLE 1. Properties of different lines of rodent kidney cells transformed by Ad5 DNA

Line[a]	Form of Ad5 DNA used for transformation	Presence of 14K in cells[b]	Ability of antiserum against tumorigenic lines to precipitate 14K[c]	Presence of sequences hybridizing to given *Hin*dIII fragment[d]	
				F	I
Tumorigenic					
945-4 } [e]	*Hin*dIII digest of *wt*	+	+ (very strong)	+	+
945-C1 } [e]	*Hin*dIII digest of *wt*	+	+ (very strong)	+	+
945-C1T } [e]	*Hin*dIII digest of *wt*	+	NT[f]	NT	NT
1019-1	*Xho*I digest of *hr6*	+	+ (strong)	+	+
1019-1SC1 } [e]	*Xho*I digest of *hr6*	+	NT	+	+
1019-C1 } [e]	*Xho*I digest of *hr6*	+	+ (strong)	+	+
1019-1T } [e]	*Xho*I digest of *hr6*	+	NT	NT	NT
1019-2	*Xho*I digest of *hr6*	−	−	+	−
1019-3	*Xho*I digest of *hr50*	−	−	−	−
972-2	*Xho*I fragment C of *wt*	−	−	−	−
972-3	*Xho*I fragment C of *wt*	−	−	−	−
983-2	*Xho*I fragment C of *wt*	−	−	−	−
983-3	*Xho*I fragment C of *wt*	−	−	−	−
Non-tumorigenic or not tested for tumorigenicity					
954-21 } [e]	*Hin*dIII digest of *wt*	+		+	−
954-C4 } [e]	*Hin*dIII digest of *wt*	+		+	−
954-5	*Hin*dIII fragment G of *wt*	−		−	−
268-C3	Sheared *wt*	−		−	−
424-C1	Sonicated *wt*	+		+	+
512-C8	*Hin*dIII fragment G of *wt*	−		−	−

[a] 424-C1 and 512-C8 are from rats; all others are from baby hamsters.
[b] Detected by immunoprecipitation with a pooled antitumor serum (see text).
[c] See text.
[d] Detected by Southern blots of *Hin*dIII-digested cell DNA (see text).
[e] Related lines, as described in the text.
[f] NT, Not tested.

gen was synthesized from an early gene block other than E1, the full expression of this block being dependent on E1 functions. As expression of E4 (between map units 91.3 and 99.3) is known to be particularly sensitive to E1A control (23), we analyzed 945-C1 and several other Ad5-transformed cell lines to determine whether the synthesis of 14K antigen correlated with the presence of E4 sequences.

Production of 14K antigen in transformed cells correlates with the presence of sequences between 89 and 97 map units of the Ad5 genome. A number of hamster cell lines transformed by different preparations of Ad5 DNA were analyzed for the production of 14K antigen. Figure 3 shows a typical result. The two lines 972-3 and 983-2, which were transformed by the left 16% of the Ad5 genome, the *Xho*I-C fragment, and therefore contain only region E1, failed to synthesize 14K antigen. This is consistent with the above suggestion that this polypeptide is not encoded by E1. In contrast, 954-21, 945-C1, and a related tumor-derived line, 945-C1T, which were all transformed by a total *Hin*dIII digest of Ad5 DNA, contained easily detectable amounts of 14K. The presence of this protein in 945-C1 cells is in agreement with the fact that antitumor sera raised against these cells in hamsters are capable of immunoprecipitating 14K.

The results of Fig. 3 and of similar analyses on other transformed lines are summarized in Table 1, in which, for those lines found to be tumorigenic in newborn hamsters, we have also indicated the efficiency with which the corresponding tumor sera precipitate 14K. It is clear from Table 1 that no cell line transformed by purified left-end fragments of Ad5 DNA contained any detectable 14K antigen or elicited any immune response against this protein, further confirming that this protein is not encoded by E1.

To determine whether the 14K antigen could be an E4 product, we analyzed the viral DNA

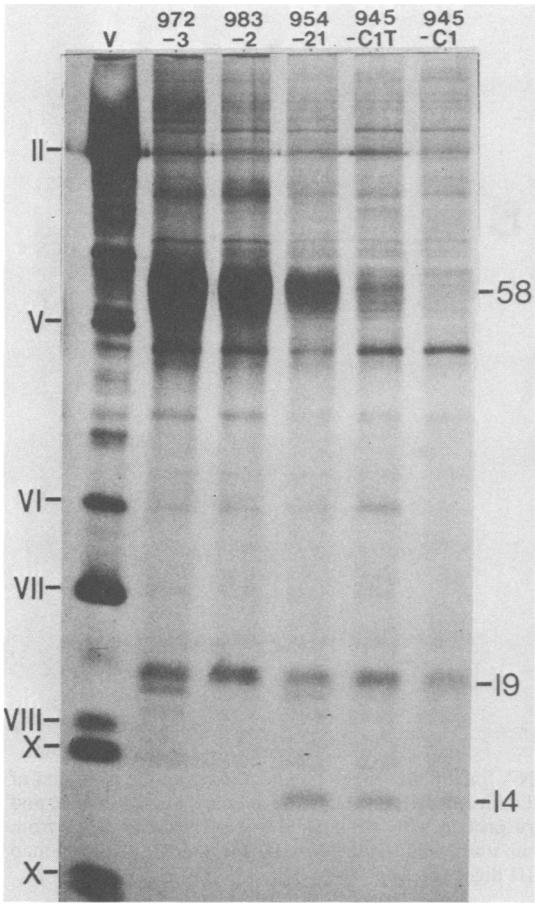

FIG. 3. Synthesis of 14K and other antigens in Ad5-transformed cells. For each line, monolayers of cells in four 150-mm plates were labeled with 100 μCi of [^{35}S]methionine per plate for 4 h. Whole-cell extracts were immunoprecipitated with the pooled serum (see text), and the precipitates were analyzed on SDS-gels. V, Virus marker.

contents of a variety of transformed lines by the Southern technique, using as probes plasmids containing either the *Hin*dIII-F (89 to 97 map units) or the *Hin*dIII-I (97 to 100) fragment of the Ad5 genome. The results are shown in Fig. 4. Lines transformed by purified DNA fragments from the left end did not contain sequences hybridizing to the right end of the genome, as expected. However, even though only the left end of Ad5 is needed for transformation, a high proportion (five of seven) of independent lines transformed by sheared, sonicated, or restricted but unfractionated Ad5 DNA contained sequences from the right end. Of these, three lines (945-4, 1019-1, and 424-C1) had viral sequences hybridizing to both F and I, and two (1019-2 and 954-21) had sequences hybridizing to F only. The results from the experiment shown in Fig. 4 and other analyses are summarized in Table 1. In addition, several lines were analyzed for the presence of sequences from region E1, using as probes plasmids containing Ad5 *Hin*dIII fragment E (8 to 17 map units) or G (0 to 8 map units). All lines contained these sequences, except 512-C8, which was transformed by purified *Hin*dIII fragment G and contained only sequences hybridizing to this region (data not shown).

The structures of mRNA species from region E4 (2, 4) indicate that the promoter and leader sequences are at about 99.2 map units and therefore fall within the *Hin*dIII-I fragment, whereas most of the coding sequences must lie between 96.8 and 91.3 map units, within the *Hin*dIII-F fragment. It is clear from Table 1 that every cell line synthesizing 14K and capable of eliciting an antigenic response to 14K contained sequences hybridizing to *Hin*dIII-F. In addition, most of these lines also contained sequences hybridizing to *Hin*dIII-I. Of the two distinct lines that contained sequences hybridizing to *Hin*dIII-F but not to *Hin*dIII-I, 1019-2 failed to produce 14K and elicited no response to it, whereas 954-21 and its subclone 954-C4 both produced 14K. These results suggest therefore that the 14K antigen is encoded by region E4, but that, depending perhaps on the way it is integrated into the DNA of the host cell, the promoter and leader sequences of this region are not always essential for its expression.

Synthesis of the 14K antigen in vitro and sequencing of its amino terminal. Nucleotide sequences have been determined for region E1 of Ad5 (3, 33) and for region E4 of the closely related Ad2 (15). It was therefore possible to ascertain whether either of these regions encodes the 14K antigen by sequencing the amino-terminal end of this polypeptide after synthesis in a cell-free system.

To demonstrate the synthesis of 14K antigen in vitro, total cytoplasmic poly(A)$^+$ mRNA from KB cells infected with *wt* Ad5 for 8 h in the presence of cycloheximide was incubated with [^{35}S]methionine in a cell-free rabbit reticulocyte system, which was then immunoprecipitated with 945-C1 or 945-4 antitumor serum. The immunoprecipitate was analyzed by SDS-polyacrylamide gel electrophoresis, together with an immunoprecipitate prepared in a similar manner from an extract of infected KB cells labeled from 4 to 8 h after infection. Figure 5 shows that the cell-free system synthesized an antigen which comigrated with the 14K polypeptide immunoprecipitated from infected cells (cf. lanes e and f). This in vitro product (see also lane c) was not precipitated by nonimmune hamster serum (lane d) nor was it synthesized in cell-free systems to

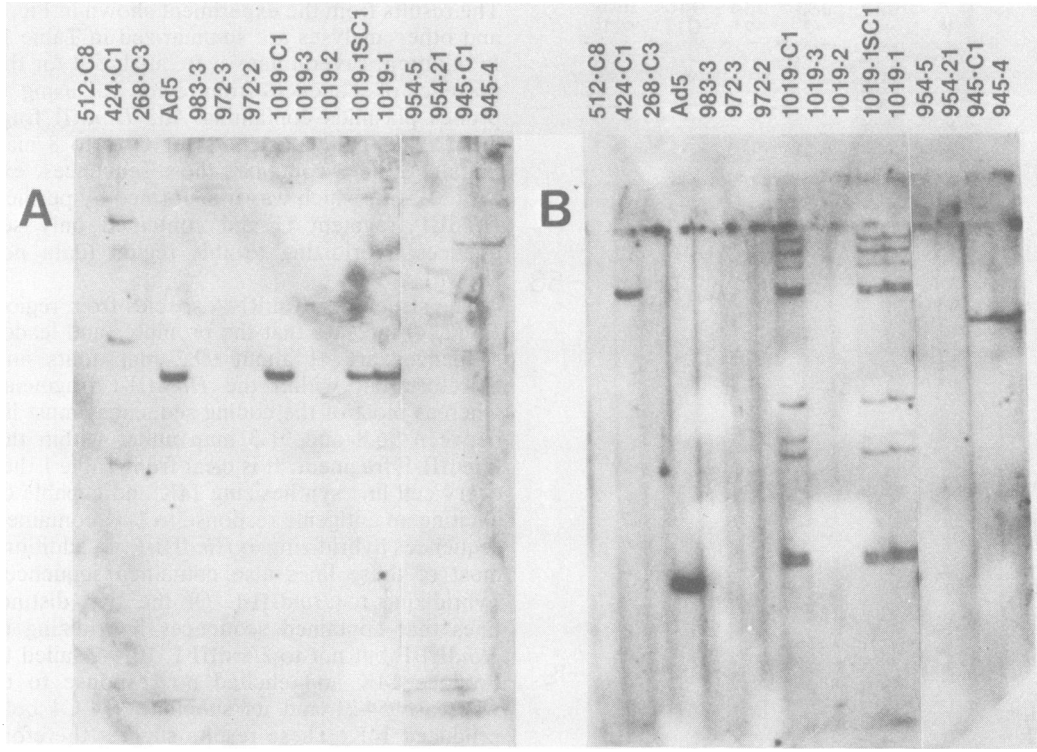

FIG. 4. Analyses of cell lines transformed by Ad5 DNA for the presence of sequences from the right end of the viral genome. DNA extracted from cells was digested with *Hin*dIII, electrophoresed on 1% agarose gels, and then transferred to nitrocellulose filters. The filters were probed with ^{32}P-labeled, nick-translated DNA from plasmids carrying (A) the *Hin*dIII-F fragment (89 to 97 map units) and (B) the *Hin*dIII-I fragment (97 to 100 map units) of Ad5 DNA. Lanes marked Ad5 contain a *Hin*dIII digest of Ad5 DNA.

which had been added either no mRNA (lane a) or mRNA from uninfected KB cells (lane b). (The nature of the other polypeptides in lanes c, d, and e, which were synthesized only with infected-cell mRNA and precipitated nonspecifically, is not known.) Results similar to these were obtained when a cell-free system from Krebs II mouse ascites cells was used in place of the reticulocyte system.

To examine the similarity of the 14K in vitro and in vivo products further, two-dimensional thin-layer maps of the tryptic peptides of each were prepared. Autoradiographs of these (Fig. 6) showed that the patterns of [^{35}S]methionine-containing peptides were identical. This result, together with the similar antigenicities and mobilities on denaturing gels, suggested that the in vitro and in vivo products were closely related, it not identical.

For amino acid sequencing, syntheses were carried out in the reticulocyte system in the presence of radioactively labeled amino acids selected from those which were likely to be present in the amino-terminal portion of a product from region E1 or E4. As described in Materials and Methods, the reaction mixtures were precipitated with 945-4 serum, the immunoprecipiates were electophoresed on SDS-gels, and material in the radioactive 14K band was extracted from each gel. This material was then processed on a Beckman automatic sequencer, and the samples recovered from each cycle were counted for radioactivity. In an initial experiment with [^{35}S]methionine, radioactivity was released in the first cycle, indicating that the 14K antigen was synthesized in vitro with an unblocked amino-terminal group. Mixtures of preparations of 14K synthesized with [^{35}S]methionine and [^{3}H]isoleucine, [^{3}H]leucine, or [^{3}H]alanine were then sequenced, and the separate counts for each isotope were determined. The results (Fig. 7) show that [^{35}S]methionine was released in cycles 1 and 19; [^{3}H]isoleucine, in cycles 2 and 16; [^{3}H]leucine, in cycles 5, 7, 13, 22, and possibly 6; and [^{3}H]alanine, in cycles 12 and 20.

Except for leucine in cycle 6, the positions of the labeled amino acids in Fig. 7 match those predicted from the nucleotide sequence of open reading frame 3 in region E4 of Ad2 (15) given at

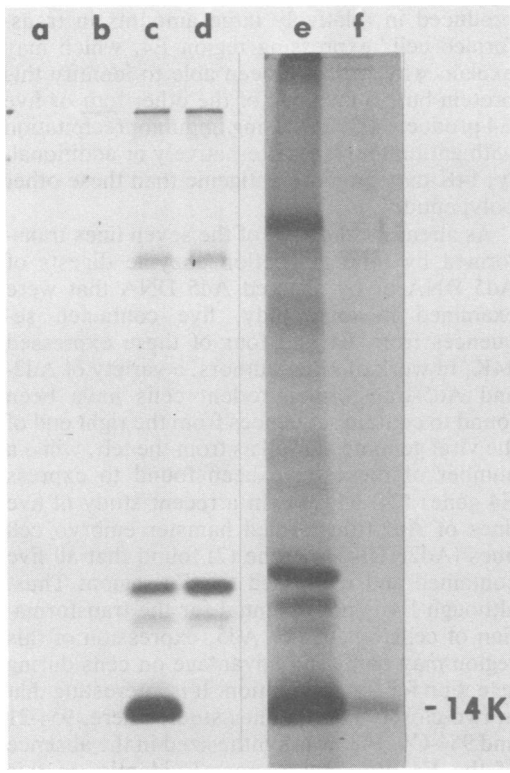

FIG. 5. Synthesis of 14K antigen in a cell-free reticulocyte system. (Lanes a to e) A rabbit reticulocyte system was incubated with [^{35}S]methionine and (a) no added mRNA; (b) total cytoplasmic poly(A)$^+$ mRNA from uninfected KB cells; (c, d, e) total cytoplasmic poly(A)$^+$ mRNA from KB cells infected for 8 h with *wt* Ad5 in the presence of cycloheximide. The incubated mixtures were immunoprecipitated with (a, b, c, e) 945-4 antitumor serum; and (d) nonimmune hamster serum, and the immunoprecipitates were analyzed on SDS-polyacrylamide gels. (Lane f) A similar analysis of antigens precipitated with 945-4 serum from KB cells infected for 8 h with *wt* Ad5 and labeled with [^{35}S]methionine 4 to 8 h postinfection.

the bottom of Fig. 7. On the other hand, the observed amino acid positions match none of the sequences predicted from any open reading frame on either DNA strand in region E1. Because of the discrepancy at position 6, the sequencing of 14K labeled with [^{3}H]leucine was repeated but with a similar result. The identity of this amino acid residue therefore remains uncertain. It may be leucine, although this would necessitate a difference of at least two bases between the codon for this amino acid in the strain of Ad5 used here and the AGG codon of Ad2 determined by Hérissé et al. (15). It seems more likely that the rather large release of radioactivity in cycle 6 represents an artifact due to the presence of leucine residues on each side of this position.

DISCUSSION

The results presented here suggest that the 14K antigen is a product of region E4 of the Ad5 genome and is encoded by the sequence from 96.7 to 95.6 map units (nucleotides 7,333 to 6,983 of Hérissé et al. [15]) on the leftward transcribed strand. This would give the polypeptide a theoretical molecular weight of 13,300. It is likely, as Hérissé et al. (15) point out, that this antigen is translated from mRNA 4a, one of the most abundant mRNAs from E4 (2, 4). This is a very large mRNA with a single splice linking the common E4 leader sequence at 99.2 to 99.0 map units to the body which extends from 96.8 to 91.3 map units. Translation would begin at the first initiation codon of this mRNA, as appears to be the general, but not invariable, rule in eucaryotic messages (3, 20).

We have found the 14K antigen to be concentrated in nuclear rather than cytoplasmic extracts of infected cells (data not shown), and it has also been located in the nucleus by immunofluorescent-antibody studies (Rowe, unpub-

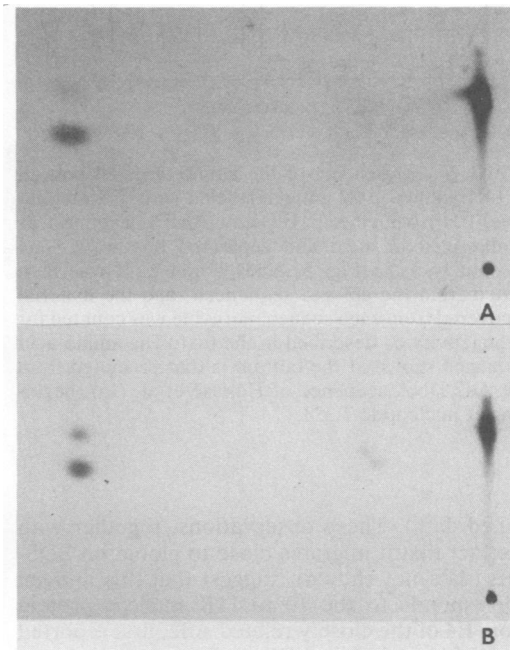

FIG. 6. Maps of [^{35}S]methionine-containing tryptic peptides of 14K antigen synthesized (A) in vivo and (B) in vitro. The 14K bands were eluted from gels similar to those in Fig. 5, lanes e and f, and digested with trypsin, and two-dimensional maps were prepared as described in the text. In each case the origin is at the lower right with electrophoresis horizontally and chromatography vertically.

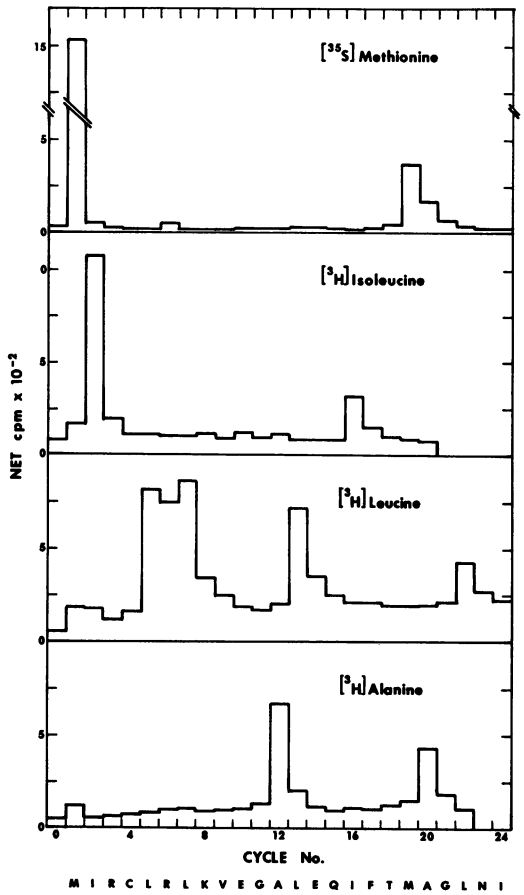

FIG. 7. Sequencing of the amino-terminal portion of 14K antigen. 14K antigen labeled with [^{35}S]methionine, [^{3}H]isoleucine, [^{3}H]leucine, or [^{3}H]alanine was synthesized in vitro and separated on an SDS-gel essentially as in Fig. 5, lanes c and e. It was then eluted from the gel and sequenced, and the material recovered from each sequencing cycle was counted for radioactivity as described in the text. The amino acid sequence shown at the bottom is that predicted from the Ad2 DNA sequence of Hérissé et al. (15), beginning at nucleotide 7,333.

lished data). These observations, together with the fact that it migrates close to globin on SDS-gels (data not shown), suggest that this antigen corresponds to the 10 to 11K nuclear protein from E4 of the closely related Ad2, first reported by Walter and Maizel (37) and since studied by a number of workers (13, 14, 22, 26, 30). This protein is made in sufficient quantity to be detected in extracts of infected cells against the background of host protein synthesis (30, 37), and it is the most abundant product when selected E4 mRNAs are translated in a cell-free system (22, 26). On this basis, it is likely to be produced in relatively large amounts in transformed cells expressing region E4, which may explain why we have been able to identify this protein but so far none of the other four or five E4 products (23, 26), using immunoprecipitation with antitumor sera. Alternatively or additionally, 14K may be more antigenic than these other polypeptides.

As already indicated, of the seven lines transformed by total restriction enzyme digests of Ad5 DNA or by sheared Ad5 DNA that were examined in this study, five contained sequences from E4 and four of these expressed 14K. In work of other authors, a variety of Ad2- and Ad5-transformed rodent cells have been found to contain sequences from the right end of the viral genome as well as from the left, while a number of these have been found to express E4 genes (24, 35, 39). In a recent study of five lines of Ad2-transformed hamster embryo cell lines (Ad2HE1–5), Esche (7) found that all five contained and expressed the E4 region. Thus, although E4 is not essential for the transformation of cells by Ad2 or Ad5, expression of this region may confer an advantage on cells during selection for transformation. It is interesting that in two closely related lines studied here, 954-21 and 954-C4, 14K was synthesized in the absence of the E4 promoter region. Evidently, in this case the coding portion of E4 must have been incorporated into cellular DNA so that it became linked to either a cellular or another viral promoter, from which transcription is initiated.

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