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Expression of the Gene Encoding the Adenovirus DNA Terminal Protein Precursor in Productively Infected and Transformed Cells

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The major product of in vitro translation of early RNA prepared from H5*ts*125-infected cells and selected by hybridization to adenoviral DNA fragments spanning the region from 14.7 to 31.5 map units has been shown to be identical to the 87-kilodalton terminal protein precursor. A 72- to 75-kilodalton polypeptide whose mRNA can be selected by DNA from this same region and made in the presence of anisomycin was indistinguishable from the 72-kilodalton single-stranded DNA-binding protein encoded by the region from 60.1 to 66.6 map units. The accumulation of cytoplasmic RNA sequences complementary to these *l*-strand genes under various conditions of infection and in certain lines of transformed cells has been investigated by solution hybridization of cytoplasmic RNA to the separated strands of restriction endonuclease fragments of adenoviral DNA. During the early phase, RNA sequences complementary to the region from 11.6 to 36.7 map units were present at a concentration of 10 to 60 copies per cell, regardless of the nature of the block used to inhibit viral DNA synthesis. By 24 h after infection in the absence of any such block, sequences complementary to the regions from 11.6 to 18.2 map units (IVa_2) and from 18.6 to 36.7 map units (E2B) accumulated to concentrations of 4,800 and 280 copies per cell, respectively. The ratio of cytoplasmic E2A RNA sequences to E2B RNA sequences remained close to 10:1 throughout the time period investigated. Of the transformed cell lines which retained E2B DNA sequences that were examined, only the T2C4 line expressed these sequences in cytoplasmic RNA. The implications of these observations for regulation of expression of the adenoviral early *l*-strand genes are discussed.

It has become apparent during the past 2 years that adenoviral early genes comprise a significantly larger set than originally appreciated; in addition to the five classical early genes (E1A, E1B, E2, E3, and E4), regions expressed before the onset of viral DNA synthesis include those that specify *l*-strand RNA sequences transcribed from 11.2 to 31.5 map units (m.u.) (27, 61), the L1 52/55k mRNA (1, 15, 16, 42, 44, 46, 58), and mRNA species encoded within the region from 11 to 35 m.u. that specify 13.5-, 13.6-, 16-, and 17-kilodalton (kd) polypeptides, whose precise locations in the genome have not been determined (41, 42a). Thus, 40 to 45% of the genetic information of the virus can be expressed during the early phase of productive infection. Although the catalog of adenoviral early genes has been expanded a great deal, we remain in considerable ignorance of the functions of most polypeptides encoded by them; several early polypeptides have, however, been implicated in viral DNA replication.

The phenotypes of host range deletion mutants that bear lesions within regions E1A and E1B suggest that sequences to the right of 3.6 m.u. in the E1A and E1B regions encode products that play some role in viral DNA synthesis (21, 34, 35). The polypeptides specified by these two early genes have been well characterized (21, 22, 29), yet nothing is known of their molecular modes of action. More significantly, there is no experimental information that permits a distinction to be made between direct participation of E1A or E1B polypeptides in viral DNA synthesis and an indirect role, such as that exhibited by mutants bearing deletions within the region from 2.6 to 3.3 m.u. in E1A; cells infected by such mutants fail to synthesize mRNA species complementary to early regions other than E1A and thus fail to replicate viral DNA (5, 21, 35).

In contrast to this inconclusive state of affairs with regard to E1A and E1B products, two early polypeptides that perform defined molecular

roles during adenoviral DNA replication have been identified. One, a single-stranded DNA-binding protein (DBP) of 72 kd (63), is encoded by E2A (40), to which the H5*ts*125 early mutation (20) has also been mapped (28). This protein appears to bind to and coat the parental single-stranded DNA displaced during each cycle of viral DNA synthesis (36); it is certainly required for elongation and may also participate in early steps during each cycle of replication (31, 62, 64). A second polypeptide that has a crucial molecular role in replication is the precursor (12, 13) to the 55-kd terminal protein that is covalently linked to the 5′ ends of DNA purified from mature adenovirions (52, 54, 55). Such a precursor (molecular weight, approximately 80,000) is found both on intranuclear viral DNA (13) and attached to genomes purified from noninfectious virions assembled in H2*ts*1-infected cells at a nonpermissive temperature (45, 61, 66). The postulate that the terminal protein precursor provides the primer for replication of linear adenoviral DNA (12, 52) has received strong support from the observation that soluble systems that replicate adenoviral DNA in vitro require DNA containing the terminal protein (11, 32, 61) and the recent demonstrations that the terminal protein precursor binds dCMP covalently in such soluble systems (43, 50).

It has been reported recently that the precursor to the terminal protein, which has an apparent molecular weight of 87,000, is translated from an mRNA species encoded by *l*-strand sequences within the region from 11.3 to 30 m.u. on the viral genome (61), a segment which includes the sites of the fourth group of mutations exhibiting a DNA-negative phenotype, the group N temperature-sensitive mutants of adenovirus type 5 (Ad5) (27, 68, 71, 72). Sequences encoding at least two additional high-molecular-weight polypeptides have also been reported to be located in this region (61). In this paper we present evidence that confirms the first observation made by Stillman and his colleagues (61) and describe the expression of this set of *l*-strand sequences (E2B) (61) under a variety of conditions of productive infection and in adenovirus-transformed cells.

MATERIALS AND METHODS

Cells and virus. HeLa cells were maintained in suspension culture in RPMI 1640 medium (GIBCO Laboratories) supplemented with 5% calf serum (Flow Laboratories, Inc.) and 10 mM glutamine. Ad2 and Ad5 virus stocks were prepared as described previously (25) and were titrated on HeLa cells as described by Williams (69). Temperature-sensitive mutants of Ad5 were obtained from J. Williams, and the H2*ts*1 mutant was obtained from J. Weber. The titers of mutant stocks were determined at both 33 and 39°C; those used in the experiments described below gave at least a 1,000-fold-lower biological titer at the nonpermissive temperature than at the permissive temperature.

Solution hybridization. The preparation of uniformly ^{32}P-labeled, single-stranded viral DNA probes and cytoplasmic RNA, the hybridization conditions, and the treatment of hybridization data were as described previously (24).

Hybridization selection. Restriction endonuclease fragments of Ad2 DNA were denatured and bound to nitrocellulose filters (Schleicher & Schuell Co.; type BA-85; 24 mm; pore size, 0.45 μm) as described by Ricciardi et al. (53), with the modifications of Cleveland et al. (18). *Hin*dIII and *Bal*I fragments of Ad2 cloned in pBR322 were gifts from S. Hsu and J. Sambrook and from S. Berget, respectively. Total cytoplasmic RNA was prepared as described previously (24) by using diethylpyrocarbonate-treated glassware and was selected on oligodeoxythymidylic acid cellulose for use in hybridization selection. The anisomycin used in some of the experiments was a gift from N. Belcher (Pfizer Inc.). Each filter was prehybridized at 42°C for 9 to 12 h in a siliconized glass scintillation vial containing 300 to 400 μl of 0.01 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4) (Sigma Chemical Co.) supplemented with 50% formamide (redistilled under reduced pressure in a nitrogen atmosphere; Fisher Scientific Co.), 0.4 M NaCl, 0.1 mg of polyadenylic acid [poly(A)] (Sigma) per ml, 0.5 mg of *Escherichia coli* tRNA (Boehringer Mannheim) per ml, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS). The prehybridization solution was removed and replaced with 200 to 400 μl of a mixture of the same composition containing 20 to 200 μl of poly(A)-containing RNA or 0.5 to 1.0 mg of total cytoplasmic RNA from infected cells instead of the poly(A). Hybridization was continued at 42°C in a temperature-controlled environmental shaker for 10 to 15 h. After hybridization, the filters were washed four times with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 6.8) containing 0.1% SDS, four times with 0.1× SSC containing 0.1% SDS (the final wash at 50°C for 2 min), and twice with 5 ml of 10 mM Tris-hydrochloride (pH 7.4) containing 2 mM EDTA at 60°C. Tightly bound RNA was eluted at 90 to 100°C for 90 s in 300 to 400 μl of distilled water. The RNA was recovered by precipitation in 10 mM Tris-hydrochloride (pH 7.4)–0.2 M potassium acetate–10 mM magnesium acetate supplemented with 3 to 4 μg of tRNA as a carrier and 2.5 volumes of ethanol. The RNA was resuspended in 20 μl of the same buffer without added carrier tRNA and reprecipitated with ethanol. The RNA pellet was dried under a vacuum to remove the residual ethanol and suspended in 6 to 10 μl of 0.5 mM EDTA (pH 7.0), and 1 to 3 μl was used to program the synthesis of proteins in the nuclease-treated reticulocyte lysate system described by Pelham and Jackson (48), which was provided by N. Cowan (Princeton University), or in a similar system purchased from New England Nuclear Corp. The former lysate contained much smaller amounts of globin mRNA than the latter (see Fig. 1). The labeled polypeptides present in 5-μl samples of the in vitro translation reaction mixture were resolved by electrophoresis in 10 or 15% SDS–polyacrylamide gels (37). After fluorography (7, 38), the dried gels were exposed to Kodak RP X-Omat or AR film at −70°C for varying times, as indicated in the figure legends.

Filters bearing separated strands. Nitrocellulose filters to which the separated strands of cloned *Hin*dIII restriction endonuclease fragments A and B were bound were prepared by a combination of the method of Ricciardi et al. (53) and the strand separation technique that we have used previously (25). Plasmid DNA containing *Hin*dIII fragment A or B of Ad2 was digested with *Hin*dIII, and the resulting fragments were resolved in a 1.4% agarose tube gel. The viral DNA band was excised from the gel and denatured in situ in 0.3 M NaOH for 20 min, and the strands were separated in a second tube gel cast in Tris-phosphate buffer (pH 7.9) and cut separately from this gel. The separated strands from four or five such gels were then transferred to nitrocellulose filters by miniature Southern blot transfer in 16× SSC (59). The separated strand filters were baked, prehybridized, and used for hybridization selection as described above for the 24-mm disk filters.

Partial proteolysis peptide maps. Comparisons of proteins by partial proteolysis in SDS followed by analysis of the peptides in 15% SDS–polyacrylamide gels were performed essentially as described by Cleveland et al. (17). The band of interest was cut from a dried, preparative SDS-polyacrylamide gel by comparison with the autoradiogram of the gel, rehydrated in 0.12 M Tris-hydrochloride (pH 6.8) containing 0.1% SDS, and loaded onto a second SDS-polyacrylamide gel containing 15% acrylamide. The gel slice was overlaid with 10 μl of *Staphylococcus aureus* V8 protease at concentrations between 25 and 50 μg/ml in standard gel sample buffer containing 0.1% SDS, and electrophoresis was performed at 30 V for 3 h to allow partial digestion to proceed in the stacking gel. Electrophoresis was then continued at 100 to 120 V until the dye front reached the bottom of the resolving gel. The peptides were visualized by autoradiography of the fluorographed gel (7, 37)

Purification and labeling of the DNA-terminal protein complex. The DNA-terminal protein complex was purified by equilibrium density gradient centrifugation in cesium chloride gradients containing guanidine hydrochloride, as previously described (52). In the case of ^{125}I-labeled preparations, purified unlabeled DNA-protein complex was iodinated in vitro with chloramine T, as previously described (51). In the case of ^{35}S-labeled preparations, 1 or 2 liters of virus-infected cells was grown in the presence of 10 mCi of [^{35}S]methionine essentially as described by Harter et al. (30) before purification of the DNA-protein complex. In all cases, piperidine was used to cleave the labeled protein from the DNA, as described previously (51).

RESULTS

Polypeptides specified by early RNA sequences complementary to the region from 14.7 to 31.5 m.u. on the adenoviral genome. Cells infected by the DNA-negative mutant H5*ts*125 synthesize the normal complement of classical early RNA sequences when they are grown at a nonpermissive temperature (4; unpublished data), as well as *l*-strand transcripts from the region from 17 to 31.5 m.u. (27). The use of such a well-characterized biological block to limit infection to the early phase should avoid any potential pitfalls of secondary effects that might be caused by drugs commonly used to block protein synthesis or DNA replication or both. The use of mutant H5*ts*125 also permits maximal accumulation of the *l*-strand transcripts of interest (which appear in the cytoplasm later than the other early species and accumulate gradually as the early phase is prolonged, as discussed subsequently) in the absence of the synthesis of abundant, late mRNA species complementary to the *r*-strand; these can be selected by hybridization of the tripartite leader segments near 16.4, 19.6, and 26.8 m.u. to DNA fragments that span the region of interest (39, 42, 53) and thus interfere with the identification of less abundant *l*-strand products. Therefore, RNA preparations from cells infected with H5*ts*125 at a nonpermissive temperature have been used in experiments to identify the gene products encoded by the region to which the group N mutations have been mapped (27, 71).

Some typical results of in vitro translation of RNA selected by hybridization to the *Hin*dIII-A (50.1 to 73.6 m.u.) and *Hin*dIII-B (17 to 31.5 m.u.) restriction endonuclease fragments of Ad2 DNA immobilized on nitrocellulose filters from poly(A)-containing H5*ts*125 early RNA are shown in Fig. 1. Figures 1A and A′ show the results of in vitro translation in a reticulocyte lysate generously provided by N. Cowan, whereas Fig. 1B illustrates results obtained with a reticulocyte lysate system purchased from New England Nuclear Corp. No hexon polypeptide II (105 kd) was detected among the polypeptides specified by unselected, poly(A)-containing H5*ts*125 RNA (Fig. 1A, A′, and B, lanes 1), indicating that late mRNA synthesis was indeed blocked by growth at 40°C, for 14 h. *Hin*dIII fragment A selected RNA specifying the 72-kd single-stranded DBP (Fig. 1A, A′, and B, lanes 2), which is encoded between 61.5 and 66.6 m.u. on the genome (14, 40). *Hin*dIII fragment B selected RNA which programmed the in vitro synthesis of a predominant 87-kd product (Fig. 1A′ and B, lanes 3). The filters used in these selections carried identical amounts of cloned viral restriction endonuclease fragments (40 μg in each case), and the translation reactions were programmed by identical fractions of the specifically selected RNA.

In this and other experiments, selection from 20 μg of poly(A)-containing H5*ts*125 RNA was sufficient to specify the relatively large amount of the 72-kd DBP observed in lane 2 (Fig. 1A, A′, and B), whereas 100 μg of the same RNA was necessary for detection of the 87-kd polypeptide observed in lane 3. This finding is in keeping with the relative concentrations of cytoplasmic RNA complementary to the E2A region

encoding the 72-kd DBP and the *l*-strand sequences between 11.6 and 36.7 m.u., as measured by solution hybridization (27) (Table 1). Furthermore, considerably longer times of exposure were necessary to detect the 87-kd product (cf. Fig. 1A and A').

In addition to the prominent 87-kd polypeptide, numerous other products of in vitro translation of *Hin*dIII-B-selected RNA were observed in different experiments. In the experiment shown in Fig. 1, bands of approximately 72, 55 to 60, 32, and 25 to 26 kd, as well as some lower-molecular-weight bands, were observed (Fig. 1B, lane 3, and Fig. 1C, lanes 2 and 3). The number and intensity of these bands varied with the RNA preparation, the batch of nitrocellulose used, and the lysate preparation (Fig. 1A' and B, lanes 3). It is not clear whether such variable polypeptide bands resulted from specific selection of complementary RNA sequences present at an even lower concentration than the mRNA encoding the 87-kd polypeptide or from nonspecifically bound RNA species whose products were amplified by the overexposure of the autoradiograms necessary to detect the 87-kd polypeptide. A polypeptide with a molecular weight of approximately 90,000 is, for example, present among the products of many translations in vitro of selected RNA (Fig. 1C, lanes 2 and 3). A 90-kd polypeptide was also present as a major product of in vitro translation of the total poly(A)-containing RNA used in the hybridization selection (Fig. 1A, lane 1) and may have appeared as a contaminant in the selected RNA by virtue of its relatively high concentration. However, the only high-molecular-weight polypeptide product consistently observed after translation of RNA selected from the H5*ts*125 early RNA preparation was that exhibiting an apparent molecular weight of 87,000.

In contrast, polypeptides of 105, 87, 75, 16 to 17, 13.5, and 13.6 kd have been described after similar hybridization selection procedures with DNA fragments that span the region from 14.7 to 30 m.u. (42, 61). In these experiments, anisomycin was used to enhance the synthesis of early

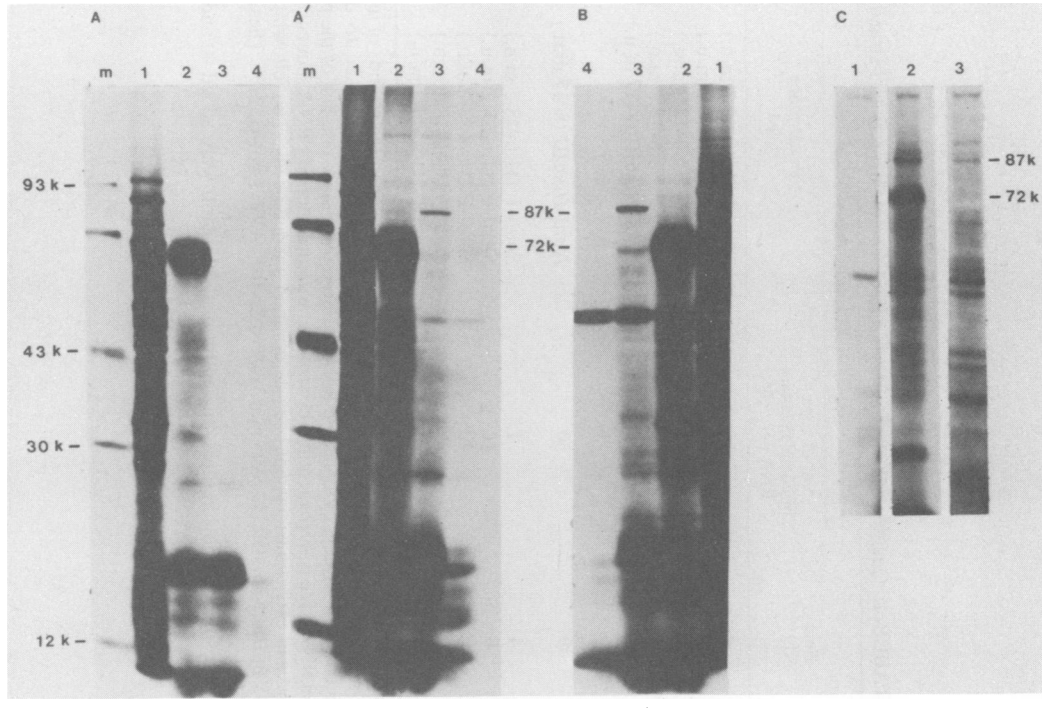


FIG. 1. In vitro translation of hybridization-selected RNA. (A, A', and B) RNA was selected by hybridization of poly(A)-containing H5*ts*125 early RNA to filters containing *Hin*dIII-A (50.1 to 73.6 m.u.) or *Hin*dIII-B (17 to 31.5 m.u.) DNA fragments and translated in vitro in a recticulocyte lysate prepared by N. Cowan (A and A') or purchased from New England Nuclear Corp. (B); exposure was for 24 h (A and B) or 48 h (A'). The polypeptides specified by poly(A)-containing Ad2 late RNA (lanes 1), *Hin*dIII A-selected RNA (lanes 2), *Hin*dIII B-selected RNA (lanes 3), or no added RNA (lanes 4) were resolved in a 12.5% SDS–polyacrylamide gel and visualized by fluorography (7). (C) *Hin*dIII-B (17 to 31.5 m.u.) DNA was used to select RNA made in the presence of 10 μM anisomycin from 3 to 7 h after infection with Ad2 (lane 2). The in vitro translation products of RNA selected by *Bal*I (14.7 to 21.5 m.u.) from H5*ts*36 early RNA (lane 3) and of no added RNA (lane 1) are shown for comparison. k, Kilodalton.

TABLE 1. Quantitation of cytoplasmic RNA complementary to *l*-strand sequences between 11.1 and 36 map units

Conditions of infection	Time (h)	IVa_2 gene region		E2B gene region	
		No. of copies of complementary RNA per cell[a]	*l*-strand probe used	No. of copies of complementary RNA per cell[a]	*l*-strand probe used
No drug	4	<1	*Sma*I-F (11.6–18.2 m.u.)	<1	*Sma*I-B (18.8–36.7 m.u.)
	6	10	*Sma*I-F (11.6–18.2 m.u.)	10	*Sma*I-B (18.8–36.7 m.u.)
	9	50–100	*Sma*I-F (11.6–18.2 m.u.)	55	*Sma*I-B (18.8–36.7 m.u.)
	24	4,800	*Sma*I-F (11.6–18.2 m.u.)	280	*Sma*I-B (18.8–36.7 m.u.)
Cytosine arabinoside[b]	7	20	*Hpa*I-*Hin*dIII (8–17 m.u.)	14	*Xho*I-F (15.9–23 m.u.)
				12	*Xho*I-G (23–27 m.u.)
				18	*Hpa*I-*Hin*dIII (17–25 m.u.)
	9	50	*Hpa*I-*Hin*dIII (8–17 m.u.)	30	*Xho*I-F (15.9–23 m.u.)
				30	*Hpa*I-*Hin*dIII (17–25 m.u.)
Cycloheximide[b]	4	1	*Sma*I-F (11.6–18.2 m.u.)	1	*Sma*I-B (18.8–36.7 m.u.)
	6	10	*Sma*I-F (11.6–18.2 m.u.)	10	*Sma*I-B (18.8–36.7 m.u.)
	9	40	*Sma*I-F (11.6–18.2 m.u.)	50	*Sma*I-B (18.8–36.7 m.u.)
H5*ts*125 (40°C)	12	ND[c]	*Sma*I-F (11.6–18.2 m.u.)	65	*Sma*I-B (18.8–36.7 m.u.)
	15	70	*Sma*I-F (11.6–18.2 m.u.)	40	*Sma*I-B (18.8–36.7 m.u.)

[a] Increasing concentrations of cytoplasmic RNA were hybridized to the separated strands of uniformly ^{32}P-labeled viral DNA restriction endonuclease fragments, and the S1-resistant, trichloroacetic acid-precipitable radioactivity was used to determine the concentrations of complementary RNA sequences from the concentration of total cytoplasmic RNA required to saturate one-half of the probe under conditions of DNA excess (24). The percent saturation of the probe in RNA excess, representing the size of the complementary RNA sequences, was determined empirically, since the RNA structure of these *l*-strand sequences at early times has not been determined unequivocally. The following values were used: *Sma*I-B, 25%; *Sma*I-F, 60%; *Hpa*I-*Hin*dIII (8 to 17 m.u.), 35%; *Hpa*I-*Hin*dIII (17 to 25 m.u.), 48%; *Xho*I-F, 42%; *Xho*I-G, 22%. These values may underestimate RNA size due to difficulty in acheiving conditions of RNA excess.

[b] Cycloheximide and cytosine arabinoside (final concentrations, 20 μg/ml) were added 40 and 60 min, respectively, after virus was added.

[c] ND, Not determined.

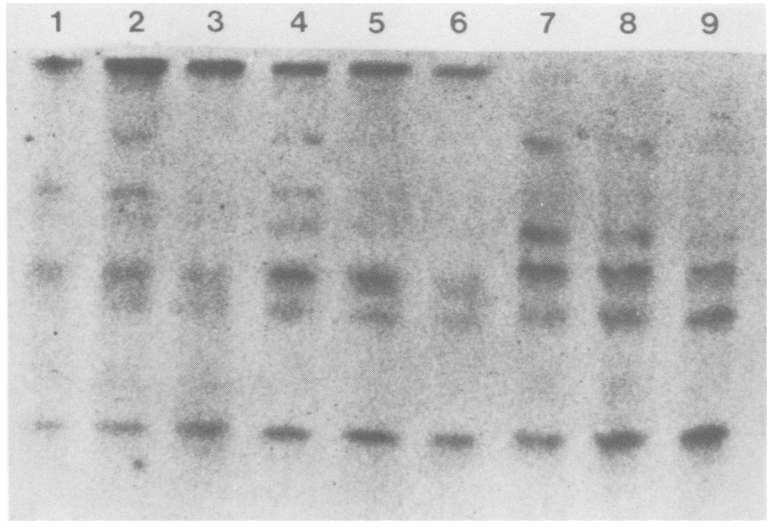
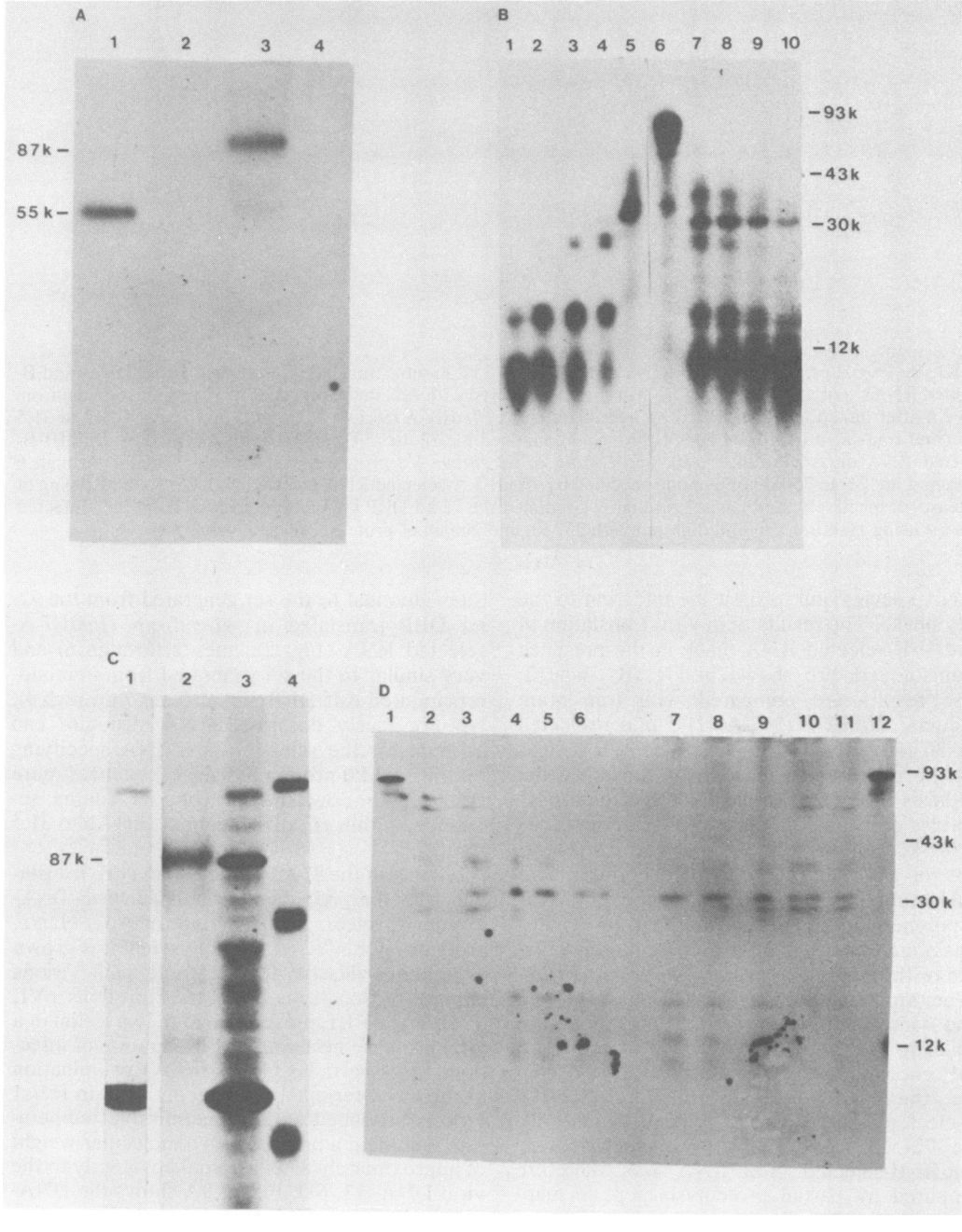


FIG. 2. Partial proteolysis comparison of the 72- to 75-kd in vitro translation products of *Hin*dIII-A- and B-selected RNAs. poly(A)-containing RNA from cells infected with Ad2 in the presence of 10 μM anisomycin from 3 to 7 h after infection was selected by hybridization to *Hin*dIII-A (50.1 to 73.6 m.u.) or *Hin*dIII-B (17 to 31.5 m.u.) and translated in vitro. Lanes 1 through 3 contained the 72- to 75-kd polypeptide specified by *Hin*dIII-B-selected RNA digested with 25, 50, and 200 ng of *S. aureus* V8 protease, respectively; lanes 4 through 6 contained the 72- to 75-kd polypeptide specified by *Hin*dIII-A-selected RNA digested with 25, 50, and 200 ng of protease, respectively; and lanes 7 through 9 contained the 72-kd DBP immunoprecipitated from Ad5-infected cells by using P-serum (56) and digested with 25, 50, and 200 ng of protease, respectively.

mRNA species and to limit the infection to the early phase. The results of in vitro translation of *Hin*dIII-B-selected RNA made in the presence of anisomycin are shown in Fig. 1C, lane 2; these results are compared with translation products of *Bal*1-E (14.7 to 21.5 m.u.)-selected H5*ts*36 early RNA, in Fig. 1C, lane 3. It is clear that the presence of anisomycin enhanced the synthesis of mRNA species that specified polypeptides of 72 to 75 and 16 to 17 kd, as well as the 87-kd polypeptide mRNA; the concentrations of both these novel polypeptides were considerably increased relative to that of the 87-kd polypeptide among the products of in vitro translation of early mRNA prepared in the presence of the drug.

The apparent molecular weight and broad band width of the 72- to 75-kd polypeptide are similar to the properties exhibited by the 72-kd DBP encoded by E2A between 61.3 and 66.6 m.u., the major translation product of *Hin*dIII-A-selected RNA (Fig. 1A, A', and B, lanes 2). The 72- to 75-kd polypeptide specified by *Hin*dIII-B-selected Ad2 RNA was therefore compared by partial proteolysis peptide mapping with the 72-kd polypeptide made in vitro from *Hin*dIII-A-selected Ad2 RNA and with immunoprecipitated 72-kd DBP from Ad5-infected cells (Fig. 2). The 72- to 75-kd polypeptide translated from RNA selected by *Hin*dIII-B DNA (17 to 31.5 m.u.) produced a set of peptides identical to the set generated from the 72-kd DBP translated in vitro from *Hin*dIII-A-selected RNA (Fig. 2, lanes 1 through 6) and very similar to the set generated from immunoprecipitated Ad5 DBP (Fig. 2, lanes 7 through 9). No previously described RNA structure can account for the selection of mRNA specifying the E2A 72-kd protein by DNA sequences more than 10 kb downstream from its coding sequence, within *Hin*dIII fragment B (17.0 to 31.5 m.u.).

Identity of the 87-kd product of in vitro translation with the precursor to the adenovirus DNA-terminal protein. A late mutant of Ad2, H2*ts*1, produces noninfectious virions when it is grown at a nonpermissive temperature; such virions contain the uncleaved precursor proteins pVI, pVII, and pVIII, presumably due to a lesion in a viral protease necessary for maturation of infectious virus particles (4, 66, 67) An examination of the DNA-terminal protein contained in H2*ts*1 virions produced at a nonpermissive temperature identified a protein with a molecular weight of approximately 87,000 bound covalently to the viral DNA (13, 61). Figure 3A shows the DNA-terminal protein released by 1 M piperidine treatment of the DNA-protein complex isolated from H2*ts*1 virions grown at 33°C (Fig. 3A, lane 1) or 39.5°C (lane 3) and labeled in vitro with ^{125}I (51). The DNA-terminal protein isolated from H2*ts*1 grown at 40°C migrated with an apparent


A
1 2 3 4
87k –
55k –
B
1 2 3 4 5 6 7 8 9 10
–93k
–43k
–30k
–12k
C
1 2 3 4
87k –
D
1 2 3 4 5 6 7 8 9 10 11 12
–93k
–43k
–30k
–12k

molecular weight of 87,000 in our gel system, compared with Ad2 virion protein standards, using the molecular weights reported by Anderson et al. (2). Isoelectric focusing of the two forms of the DNA-terminal protein indicated that the 87-kd form is more basic than the 55-kd form by approximately 0.9 pH units; the pIs are 6.85 and 5.95, respectively (Rekosh, unpublished data).

The 55- and 87-kd forms of the terminal protein were compared by partial proteolysis with *S. aureus* V8 protease (17) (Fig. 3B). Both proteins yielded a doublet of approximately 26 to 27 kd, which was sensitive to further proteolysis, and a doublet of approximately 14 to 16 kd, which appeared to be more resistant to further digestion. Small partial products of 9 to 11 kd which were not resolved into discrete bands were generated by digestion of both proteins. The 87-kd form of the protein yielded a partial proteolysis product of approximately 31 kd, which was relatively resistant to further proteolysis and was not found among the products of digestion of the 55-kd protein. A second unique low-molecular-weight peptide (molecular weight, approximately 13,000) was generated by digestion of the 87-kd protein. The peptide patterns produced by digestion of these two proteins are consistent with the precursor-product relationship of the 87- and 55-kd forms suggested by the isolation of the 87-kd protein from the H2*ts*1 processing mutant.

The 87-kd terminal protein precursor prepared from H2*ts*1 virions comigrated in a 10% SDS–polyacrylamide gel with the major product of in vitro translation of early mRNA selected by hybridization to *Hin*dIII fragment B (Fig. 3C). The 87-kd polypeptide made in vitro from *Hin*dIII-B-selected Ad2 early RNA was compared by partial proteolysis in SDS with the H2*ts*1 terminal protein labeled in vivo with [^{35}S]methionine. At the lowest concentration of protease used, a doublet of 61 to 66 kd, a faint doublet of 37 to 39 kd, and a set of three bands in the range from 26 to 31 kd were generated from both the authentic terminal protein precursor (Fig. 3D, lanes 1 through 6) and the 87-kd polypeptide made in vitro (Fig. 3D, lanes 7 through 12). As observed during digestion of the ^{125}I-labeled 87-kd protein (Fig. 3B), the 26- to 27-kd peptides were sensitive to further proteolysis, whereas the 31-kd peptide was relatively resistant. Low-molecular-weight peptides (molecular weights, approximately 16,000, 15,000, 13,000, and 9,000 to 11,000) were products of more extensive digestion of both proteins. A slight variation in the mobility of the peptide of approximately 27 kd relative to the 31-kd peptide appeared to be the only difference between the patterns of peptides produced upon partial digestion of these two proteins. Whether this difference reflects some modification of the protein synthesized in cells that does not take place in the in vitro translation reaction is not clear. It should be noted that removal of the DNA from the virion protein by piperidine treatment leaves no nucleotide or phosphate attached to the protein, which might otherwise account for the mobility difference in one peptide (10), but it has not been shown that piperidine treatment does not alter the protein in some other manner (51). Stillman and co-workers compared these same two 87-kd proteins by using a reverse-phase high-pressure liquid chromatography analysis of ^{35}S-labeled tryptic peptides and concluded that the two are identical (61).

Selection of RNA by hybridization to separated DNA strands. Filters containing the separated strands of *Hin*dIII fragments A and B of Ad2 DNA were used to select complementary RNA

FIG. 3. Partial proteolysis peptide comparison of the 87-kd terminal protein precursor with the 87-kd in vitro translation product and the 55-kd terminal protein. (A) DNA-terminal protein complex was isolated from H2*ts*1 virions grown at 33 or 39°C and iodinated in vitro (51). Samples of each preparation were incubated with and without piperidine to cleave the DNA-protein linkage. H2*ts*1 was grown at 33°C with (lane 1) and without (lane 2) piperidine and also at 39°C with (lane 3) and without (lane 4) piperidine. (B) Partial proteolysis of ^{125}I-labeled 55-kd terminal protein from Ad2 virions and ^{125}I-labeled 87-kd H2*ts*1 terminal protein. Lanes 1 through 5 contained the 55-kd protein digested with 500, 200, 100, 50, and 0 ng of *S. aureus* V8 protease, respectively, and lanes 6 through 10 show the results of digestion of the 87-kd ts1 protein with 0, 50, 100, 200, and 500 ng of protease, respectively. After iodination (see text) each labeled polypeptide was subjected to electrophoresis in SDS-polyacrylamide gels, and the 55- and 87-kd proteins were excised and digested as described in the text. (C) Iodinated 87-kd protein isolated from Ad2 ts1 virions produced at 39°C (lane 2) compared with the 87-kd product of in vitro translation of *Hin*dIII-B-selected RNA (lane 3). The products of in vitro translation in the absence of added RNA are shown in lane 1, and the molecular weight markers are shown in lane 4. (D) The bands corresponding to the 87-kd in vitro translation product and the in vivo ^{35}S-labeled terminal protein from H2*ts*1 virions produced at 39°C were excised from a preparative 10% SDS–polyacrylamide gel, applied to a 15% resolving gel, and overlaid with increasing amounts of *S. aureus* V8 protease. Lanes 1 through 6 contained the 87-kd H2*ts*1 virion protein digested with 0, 25, 50, 100, 150, and 250 ng of protease, respectively, and lanes 7 through 12 contained the 87-kd in vitro translation product digested with 250, 150, 100, 50, 25, and 0 ng of protease, respectively. Proteolysis was allowed to proceed for 3 h at 30 V in the stacking gel. k, Kilodalton.

by hybridization to preparations of poly(A)-containing RNA isolated 20 h after infection with Ad2 with no block to DNA synthesis. The polypeptides specified by RNA selected by an *Hin*dIII-A *l*-strand filter are shown in Fig. 4, lane 3. The 72-kd DBP was the predominant in vitro product, as expected (40). Polypeptides specified by RNA selected by the *Hin*dIII-B *l*-strand and *r*-strand are shown in Fig. 4, lanes 4 and 5, respectively. Numerous background bands were present, but these bands exhibited similar mobilities and intensities in both lanes. The only unique polypeptide observed in lane 4 was a polypeptide of approximately 87 kd. No *Hin*dIII-B *r*-strand-specific polypeptides have been detected in these experiments, although

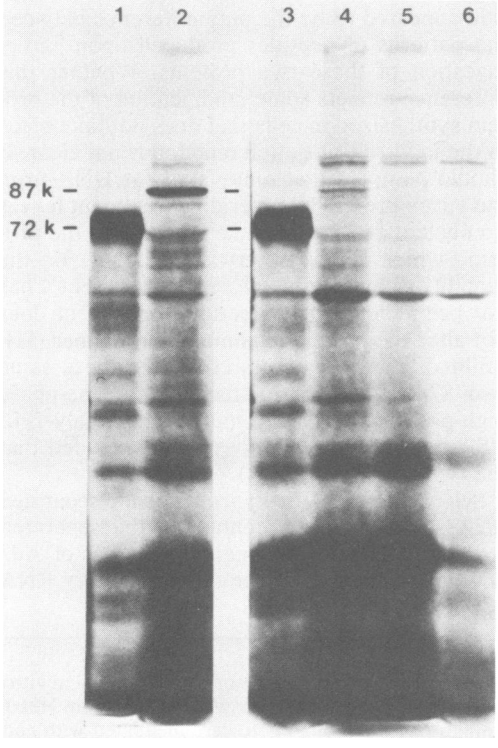


FIG. 4. Hybridization selection with separated strands of cloned viral restriction endonuclease fragments. The strands of cloned Ad2 *Hin*dIII fragments A (50.1 to 73.6 m.u.) and B (17 to 31.5 m.u.) were separated as described in the text, transferred separately to nitrocellulose filters, and hybridized to RNA isolated from cells infected with Ad2 for 24 h in the absence of drugs. Shown are the products of in vitro translation of RNA selected by hybridization to the *Hin*dIII-A *l*-strand (lane 3), the *Hin*dIII-B *l*-strand (lane 4) and *r*-strand (lane 5), and of no added RNA (lane 6). For comparison, lanes 1 and 2 show the in vitro translation products of RNA selected from early H5*ts*125 RNA by the *Hin*dIII-A and -B double strands, respectively. k, Kilodalton.

the mRNA specifying the 52,55-kd protein encoded between 29.1 and 33.3 m.u. should be selected by the *r*-strand of this region (15, 41, 44). The presence of this polypeptide may be obscured by an endogenous lysate band at a similar position in the gel. For comparison, the in vitro translation products of RNA selected from H5*ts*125 early RNA by the *Hin*dIII-A and -B double strands are shown in Fig. 4, lanes 1 and 2. Hybridization selections from H5*ts*125 early RNA by using separated strands of *Hin*dIII-B DNA confirmed the presence of mRNA specifying an 87-kd protein complementary to the *l*-strand of *Hin*dIII fragment B in the cytoplasm at early times after infection (data not shown).

Cytoplasmic accumulation of early *l*-strand RNA sequences. The steady-state concentration of cytoplasmic RNA complementary to *l*-strand sequences between 11 and 36 m.u. has been determined by hybridization in solution to the separated strands of appropriate restriction endonuclease fragments of uniformly ^{32}P-labeled Ad2 DNA. The set of probes employed permit measurement of the concentrations of cytoplasmic RNA sequences complementary to the 3′ (11 to 18 m.u.) and 5′ (18 to 30 m.u.) portions of the E2B region (Table 1).

Cytoplasmic RNA sequences encoded by *l*-strand sequences between 11 and 36 m.u. were barely detectable until 6 h after infection. The RNA sequences complementary to the *l*-strand of *Sma*I fragment B (18.8 to 36.7 m.u.), includ- the region to which the H5*ts*36 mutation has been mapped (27), increased from approximately 10 to 55 copies per cell between 6 and 9 h after infection in the absence of drugs. RNA preparations from cells infected in the presence of cytosine arabinoside to block DNA replication or in the presence of cycloheximide to block both protein synthesis and DNA replication also contained between 10 and 50 copies per cell. Essentially the same level (40 to 65 copies of RNA sequences complementary to the *l*-strand of *Sma*I fragment B per cell) was observed in two separate infections with H5*ts*125 at a nonpermissive temperature. Infection in the presence of cycloheximide (added at 40 min after infection) did not result in any increase in concentration of these *l*-strand sequences compared with infections in the absence of drugs (Table 1).

The highest level of accumulation of RNA complementary to the *l*-strand between 18.8 and 36.7 m.u. was observed after 24 h of infection in the absence of any block to DNA replication, when a value of 280 copies per cell was attained. This value does not represent a high concentration relative to the other early RNAs, which continued to accumulate late in infection, or relative to the major late transcripts; cytoplas-

mic RNA sequences complementary to E2A are present at levels of approximately 3,000 copies per cell by 24 to 32 h after infection (24, 71), whereas RNA sequences complementary to the *r*-strand between 58.5 and 70.7 m.u. (L3 and part of L4) accumulate to a concentration of 114,000 copies per cell by this same late time after infection (24).

Cytoplasmic RNA sequences complementary to the *l*-strand of *Sma*I-F (11.6 to 18.2 m.u.), the region to which the IVa_2 gene has been assigned (14, 40), were present early in infection at levels comparable to those of the *l*-strand sequences between 18 and 36 m.u. (that is, between 10 and 50 copies per cell) (Table 1). Following DNA replication, however, the pattern of accumulation of cytoplasmic RNA sequences transcribed from the IVa_2 region of the genome diverged dramatically from that exhibited by E2B sequences; 24 h after infection, IVa_2 RNA sequences were present at a level of approximately 4,800 copies per cell, whereas RNA sequences complementary to the 18- to 36-m.u. region were represented at a level of only 280 copies per cell. Such an accumulation of IVa_2 RNA sequences is similar to that observed for early region E2A, which is represented by approximately 3,400 copies of complementary RNA per cell by 24 h after infection (71).

Expression of the E2B gene region in rodent cells transformed by group C adenoviruses. Many of the rodent cell lines established after transformation by Ad2 or Ad5 whose contents of viral DNA sequences have been examined do not contain sequences homologous to the region to which the H5*ts*36 mutation has been mapped (23, 33, 57, 65). However, among others, the F4 and T2C4 lines of Ad2-transformed rat embryonic fibroblasts (26) and the HT14B and HT14A lines of H5*ts*14-transformed hamster embryonic fibroblasts (70) do contain integrated copies of this region of the adenoviral genome (26, 57). The expression of adenoviral genetic information in cytoplasmic mRNA has been examined in each of these four cell lines (23–25), but a reexamination seemed prudent, given the original failure to detect expression of the E2B gene region in productively infected cells. The expression of such sequences from integrated DNA might carry interesting implications for the mechanism of their transcription.

Previous assays for adenoviral RNA sequences employing hybridization of unlabeled, cytoplasmic RNA to the separated strands of restriction endonuclease fragments of ^{32}P-labeled Ad2 or Ad5 DNA failed to detect any RNA sequences complementary to the *l*-strand of *Hpa*I fragment C (4.5 to 25.0 m.u.), which includes sequences of the E2B region in either HT14A or HT14B cells (23, 24). Figures 5A through C show the results of hybridization of HT14B cytoplasmic RNA to additional, smaller fragments that span the region from 8.0 to 26.5 m.u. in the type C adenoviral genome, *Xho*I fragment F (22.0 to 26.5 m.u.), and the two fragments from 8.0 to 17.0 m.u. and from 17.0 to 25.0 m.u. generated by digestion of *Hpa*I fragment C (4.5 to 25.0 m.u.) with *Hin*dIII. No RNA sequences complementary to the *l*-strands of any of these fragments were detected in preparations of HT14B cytoplasmic RNA that contained sequences complementary to the *r*-strand of early region E1B (Fig. 5). Similar results were obtained when HT14A cytoplasmic RNA was examined by hybridization to this same set of *l*-strand DNA segments (data not shown). Thus, neither HT14B nor HT14A cells express the E2B region or indeed sequences of the IVa_2 gene (included within the *l*-strand of the 8.0- to 17.0-m.u. fragment), even though adenoviral DNA sequences homologous to both of these genes are integrated into the hamster genome in these cell lines (57).

In the original analysis (23, 24) T2C4 Ad2-transformed rat embryo fibroblasts were observed to contain some RNA sequences complementary to the *l*-strand of *Eco*RI fragment A (0 to 58.5 m.u.) on the Ad2 genome. Figures 5D through F show some of the results obtained when T2C4 cytoplasmic RNA was hybridized to separate strands of smaller restriction endonu-

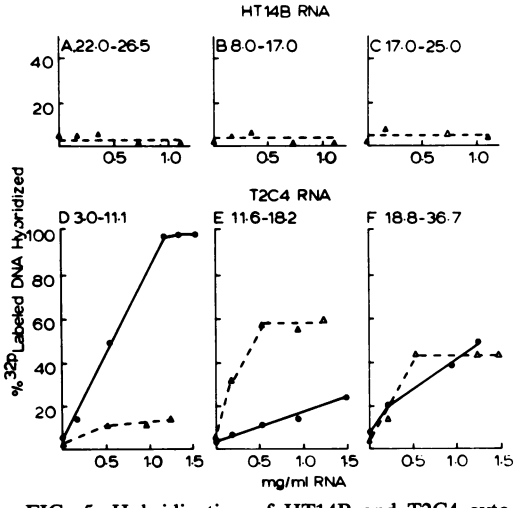


FIG. 5. Hybridization of HT14B and T2C4 cytoplasmic RNAs to the separated strands of Ad2 DNA fragments from the region from 3.0 to 36.7 m.u. The preparation of cytoplasmic RNA from transformed cells and of separated strands of restriction endonuclease fragments of ^{32}P-labeled Ad2 DNA, hybridization, and the assay of hybridization by nuclease S1 digestion were performed as described previously (25, 27). Symbols: △, hybridization to *l*-strand DNA; ●, hybridization to *r*-strand DNA.

clease fragments that included the region from 11 to 36.7 m.u. in the viral genome. As expected from the results of previous experiments, T2C4 cytoplasmic RNA preparations contained sequences complementary to the entire *r*-strand of *Sma*I fragment E (3.0 to 11.1 m.u.) (Fig. 5D). In addition, T2C4 cytoplasmic RNA saturated approximately 55 and 40% of the *l*-strands of *Sma*I fragments F and B, respectively (Fig. 5E and F). The sequences complementary to the *l*-strand of *Sma*I fragment F (total, approximately 4 m.u.) appeared to correspond to the sequences of the IVa_2 gene, which has been mapped from 15.8 to 11.3 m.u. on the *l*-strand (14, 16, 39). The sequences complementary to the *l*-strand of *Sma*I fragment B (apparently comprising some 7 m.u. in total) lie within the region to which the H5*ts*36 mutation (27) and sequences encoding the 87-kd terminal protein precursor (see previous sections) (61) have been mapped. Interestingly, T2C4 cells also contain cytoplasmic RNA sequences complementary to the *r*-strands of *Sma*I fragment F (11.6 to 18.2 m.u.), which includes the major late promoter site (73) and *Sma*I fragment B (18.8 to 36.7 m.u.), albeit at lower concentrations than the sequences complementary to the *l*-strands of these DNA fragments (Fig. 5E and F). Some RNA sequences complementary to the *l*-strand of *Sma*I fragment E (3.0 to 11.1 m.u.), whose *r*-strand includes region E1B, also appeared to be present in T2C4 cytoplasmic RNA (Fig. 5D).

DISCUSSION

Sequences lying in the *l*-strand of the adenoviral genome between 14.7 and 28.5 m.u. (*Bal*I fragments E and D, region E2B, following the terminology of Stillman et al. [61]) are expressed during the early phase of productive infection as mRNA whose predominant product when translated in vitro is a polypeptide exhibiting an apparent molecular weight of 87,000 (Fig. 1 and 4). Comparisons of migration in SDS-polyacrylamide gels and of the products of partial proteolytic digestion indicate that the E2B 87-kd polypeptide synthesized in vitro is identical to the authentic terminal protein precursor purified from H2*ts*1 virions (Fig. 3). We therefore conclude that this set of *l*-strand sequences encodes the precursor to the terminal protein, confirming a recent report by Stillman et al. (61).

In addition to the 87-kd terminal protein precursor, polypeptides of 105, 75, 16 to 17, 13.5, and 13.6 kd have been mapped within the region from 11 to 32 m.u. on the adenoviral genome (41, 42, 61). These assignments are based on the results of in vitro translation of early mRNA, usually prepared from cells infected in the presence of anisomycin and selected by hybridization to specific fragments of viral DNA. Similarly, we have observed polypeptides of 72 to 75 and 16 to 17 kd as specific products of translation of early mRNA purified by hybridization to *Hin*dIII fragment B (17.0 to 31.5 m.u.) (Fig. 1). Polypeptides of 13.5 and 13.6 kd would be poorly resolved in the gel systems which we used. On the other hand, any polypeptides of higher molecular weight than the terminal protein precursor should be clearly visible, but none (for example, the 105-kd polypeptide described by Stillman et al. [61]) was detected in any of the experiments that we performed. Furthermore, the 72- to 75-kd polypeptide whose mRNA can be selected by hybridization to *Hin*dIII fragment B appears to be identical to authentic E2A-encoded DBP by the criterion of partial proteolytic digestion (Fig. 2). Although we cannot formally exclude the possibility that the 75-kd polypeptide described by Stillman et al. (61) is a unique product of region E2B, such a novel 75-kd polypeptide would have to represent a relatively low proportion of the total protein in our experiments to escape detection in comparisons like that shown in Fig. 2.

Regardless of the mechanism used to inhibit viral DNA replication, mRNA sequences complementary to the E2B region are expressed at low levels. For example, at 9 h after infection in the absence of drugs, E2B sequences are present at a concentration of about 50 copies per cell, whereas sequences complementary to the classical early regions E1A and E2A accumulate to levels of 400 and 800 to 900 copies per cell, respectively (Table 1) (71). Moreover, the accumulation of E2B RNA sequences in the cytoplasm of infected cells appears to be indifferent to inhibition of protein synthesis to 95% or to the H5*ts*125 mutation (Table 1), two parameters that lead to accumulation of classical early RNA sequences, including E2A (8, 9, 19, 42, 47, 49). These results come as something of a surprise in light of the structures and mode of expression of E2B mRNA species deduced from the results of heteroduplex analyses (61); the three E2B mRNA species described carry the 5′-terminal leader segments encoded near 68.6 and 75.0 m.u. on the *l*-strand characteristic of E2A mRNA (15, 16) and are therefore believed to be transcribed from the E2 early promoter site located near 75.0 m.u. (3). In this context, it is also interesting that the ratio of E2A cytoplasmic RNA sequences to E2B cytoplasmic RNA sequences remains constant at close to 10:1 between 9 and 24 h after infection, although the absolute amounts of both sets of sequences increase approximately fivefold (Table 1). If the E2A and E2B mRNA species are indeed derived by differential processing of one primary product of transcription extending from the E2 promoter site near 75.0 m.u. to or beyond the E2B

polyadenylation site at 11.3 m.u. (61), then this observation implies that such processing events are not subject to temporal regulation, another apparent difference between this and other early regions (15, 16, 21, 60) and between this *l*-strand transcriptional unit and the major late *r*-strand unit (46).

In the absence of viral DNA synthesis, the RNA sequences complementary to the *l*-strands of the IVa_2 gene region and E2B (*Sma*I fragments F and B, respectively [Table 1]) are present at a ratio of 1:1, consistent with the structures of early E2B-complementary mRNA species reported by Stillman et al. (61). However, by contrast to the constant ratio of E2A to E2B cytoplasmic RNA sequences, the ratio of IVa_2 sequences to E2B sequences increases dramatically with the onset of viral DNA synthesis, to a value of approximately 20:1 by 24 h after infection. Whether this substantial accumulation of IVa_2 mRNA sequences reflects selective processing of the *l*-strand primary product of transcription, as discussed above, or, more probably (3), activation of an IVa_2 promoter site is currently under investigation. Our understanding of expression of these *l*-strand early genes is further confounded by the selection of functional mRNA specifying the 72-k DBP by E2B DNA sequences located more than 10 kb downstream from the E2A coding region (Fig. 1 and 2); such an mRNA species must include the normal E2A sequences linked in some as-yet-undefined fashion to E2B sequences. It also seems that uncommon or perhaps aberrant modes of processing are enhanced in the presence of anisomycin, a stringent inhibitor of protein synthesis, for the ratios of the mRNA species encoding the 87-kd terminal protein precursor and the 72-kd DBP are altered quite dramatically in favor of the latter when this drug is included in the culture medium (Fig. 1A′ and B, lanes 3, and Fig. 1C, lane 2).

Although Ad5 group N mutant viruses fail to initiate transformation of rodent cells under some conditions of infection at a nonpermissive temperature (71, 72) the mutations themselves all lie to the right of the viral genes whose products appear to be necessary for maintainence of the transformed cell phenotype (22). Therefore, it is no surprise that many lines of rodent cells established after transformation by type C adenoviruses do not retain sequences homologous to the E2B region (57, 65) and that only one of the lines possessing these sequences that we examined (T2C4) expresses them in cytoplasmic RNA (Fig. 5). Moreover, the argument can be made that the E2B sequences, although they can be expressed (Fig. 5) (42a), contribute little to the phenotype of T2C4 cells, for a more recent analysis of the viral DNA sequences present in T2C4 cells failed to detect any sequences homologous to the viral genome to the right of position 17 (65). This difference presumably reflects the different passage history of the cells, for the T2C4 cells that we examined are direct descendents of those originally analyzed by Sambrook et al. (57), who reported the presence of DNA sequences homologous to the E2B region. As it seems most likely that some sequences in the region from 24.2 to 58.5 m.u. are absent from T2C4 cells, it may be that the E2B region in these cells is expressed from a novel promoter site or from viral sequences permuted with respect to their arrangement on the adenoviral genome. Evaluation of these possibilities requires knowledge both of the organization of the integrated adenoviral DNA sequences in T2C4 cells and of the structure of the E2B mRNA species made, neither of which has yet been investigated.

Finally, it must be noted that although the mRNA encoding the 87-kd terminal protein precursor spans the region to which the Ad5 group N mutations have been mapped (27, 71), there is no evidence that a thermolabile terminal protein precursor is responsible for the failure of such mutants to replicate their DNAs at a nonpermissive temperature, attractive as this deduction seems. Until the coding sequences of the polypeptides specified both by the E2B mRNA and the other mRNAs assigned to this general region of the genome (41, 42) are identified precisely, the possibility that the group N mutations define a viral polypeptide other than the 87-kd terminal protein precursor which is essential to DNA synthesis must remain open.

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