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Transformation-Deficient Adenovirus Mutant Defective in Expression of Region 1A but Not Region 1B

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An adenovirus type 5 host range mutant (*hr*440) has been isolated which is defective in a splicing event required to generate the middle-sized mRNA from early region 1A. This defect has been ascribed to two adjacent nucleotide changes which lie five and six nucleotides from the 5′ splice site for this mRNA (Solnick, Nature **291**:508–510, 1981). One of these changes introduces an amber codon into the reading frame of the largest region 1A mRNA, resulting in the production of a truncated polypeptide. Like other region 1A mutants, *hr*440 is defective in the production of mRNA from early regions 2 and 3, but *hr*440 is unusual in that transcription from regions 1B and 4 is normal. Furthermore, although region 1B expression is unaffected, *hr*440 does not transform baby rat kidney cells. Therefore, expression of early region 1B is insufficient for transformation, eliminating the possibility that region 1A is required only to induce such expression.

The adenovirus genome contains at least 10 transcriptional units whose activity during the viral lytic cycle is temporally regulated in a complex fashion. Although little is known concerning the mechanisms of this regulation, combined genetic and biochemical studies have shown that in some cases the activity of one region of the viral genome is regulated by the activity of another. For example, a mutant (*ts*125) with a lesion in early region 2 (E2) maintains elevated transcription from E4 during infection at the nonpermissive temperature, whereas E4 expression decreases rapidly 6 h after a wild-type infection (30). Such results suggest that a product of E2, perhaps the 72,000-dalton (72K) DNA binding protein, is a negative regulator of transcription. Pleiotropic effects are also produced by mutations in E1A; in this case, expression of E1B, E2, E3, and E4 is substantially decreased compared with wild-type levels (3, 24, 28), suggesting that one or more products of E1A may orchestrate the initial stages of infection.

Although nearly the entire genome is required for a productive infection of permissive human cells, E1A and E1B are sufficient for the transformation of nonpermissive rodent cells (15, 39). It is reasonable to speculate that E1A plays a regulatory role in transformation that is in some way similar to its role in the infectious cycle. One possibility is that E1A gene products induce the expression of cellular genes that are responsible for altering cellular growth properties. Indeed, the overproduction of cellular gene products has been invoked to explain the transforming activity of several RNA tumor viruses (6). Another possibility is that in transformation as in infection, E1A is required to induce the expression of E1B, and E1B then plays a direct role in producing a transformation phenotype. This postulate, which was first proposed by Berk et al. (3), is consistent with the observation that the 58K protein from E1B is the predominant tumor antigen (38).

In this paper we describe an E1A mutant of adenovirus type 5 (Ad5) which exhibits an unusual pattern of early expression: E1B and E4 are expressed normally, whereas E2 and E3 are expressed only at greatly reduced levels. Despite normal expression of E1B, however, the mutant is defective for transformation of rat cells. Therefore, E1B expression is insufficient for transformation, eliminating the possibility that E1A is required only to induce such expression.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of cell lines 293 and HeLa were grown in Dulbecco modified Eagle medium (Microbiological Associates) supplemented with 10% calf serum, 100 μg of streptomycin per ml, and 100 μg of penicillin per ml. Suspension cultures of HeLa cells were grown in the same medium supplemented with 5% calf serum. Ad5, Ad2, and *dl*309 were propagated on HeLa monolayers, and *hr*440, *hr*A, *dl*A, and *dl*312 were propagated on line 293 cells. *dl*309 and *dl*312 were kindly provided by T. Shenk. Viral

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infections and titrations were performed essentially as described by Williams (41). Unless otherwise noted, a multiplicity of 5 to 10 PFU/cell was used for all infections.

Isolation of viral DNA and RNA. Adenoviral DNA was isolated from virions purified on a CsCl gradient, essentially as described by Jones and Shenk (22). DNA fragments generated by cleavage with restriction endonucleases (Bethesda Research Laboratories) were separated on agarose gels and electroeluted, as previously described (36).

Cytoplasmic RNA was prepared from infected HeLa suspension cells by using the Nonidet P-40 lysis procedure of Berk et al. (3).

S1 analysis. S1 analysis was performed by the method of Berk and Sharp (4), except for the modification described by Favaloro et al. (11). Unlabeled rather than labeled DNA fragments were used for hybridization to RNA, and S1-treated hybrids fractionated on agarose gels were visualized by blotting onto nitrocellulose and hybridizing a nick-translated probe generated from Ad5 DNA.

Hybridization selection and in vitro translation of RNA. Using the methods described and cited by Lewis and Mathews (29), cytoplasmic RNA was hybridized to DNA fragments bound to nitrocellulose filters, and after extensive washing the RNA was eluted from the filters by heating to 100°C in water. Selected RNA was translated with a rabbit reticulocyte lysate pretreated with micrococcal nuclease. Proteins synthesized in the presence of [^{35}S]methionine were fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gels by the method of Laemmli (25) (except for the use of a 5% stacking gel rather than a 3% stacking gel), and the gels were processed for fluorography (7).

Protein labeling and immunoprecipitation. Monolayer cultures of primary baby rat kidney cells growing on 6-cm dishes were labeled in medium containing 1/20 the normal amount of unlabeled methionine, 100 μCi of [^{35}S]methionine (1,000 Ci/mmol), and 2% calf serum. The cells were lysed with Nonidet P-40 and centrifuged briefly, and the resulting supernatant was used for immunoprecipitation by the methods described by Crawford et al. (9). 14B antiserum (provided by R. Frisque) was obtained from hamsters bearing tumors induced by HT14b, an Ad5-transformed line of hamster embryo cells which expresses only E1 (12). Immune complexes adsorbed to formaldehyde-fixed *Staphylococcus aureus* were eluted by boiling in sample buffer (25), and immunoprecipitated proteins were fractionated by electrophoresis through SDS-polyacrylamide gels.

Transformation assays. Primary cultures of rat kidney cells were prepared by dispase-collagenase dispersion (Boehringer Mannheim) of kidneys from 6-day-old Fisher rats. At 2 days before infection, 6-cm culture dishes were seeded with 3.5×10^5 cells, and the cultures were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum, with a change of medium every 4 days. Dense foci were scored 4 weeks after infection.

With the following modifications, DNA infections were performed by using the $CaCl_2$ precipitation method of Graham and Van der Eb (17). Precipitated DNA was added directly to the culture medium, which was then replaced with fresh medium 7 h later. DNA from rat cells was used as a carrier.

RESULTS

To generate mutants with lesions only in E1A, we used a region-specific mutagenesis technique (35). This method employed a phenotypically wild-type variant of Ad5 (*dl*309) (23) which contains a single *Xba*I site within E1A, near the conventional left end of the viral genome (map position 3.7). The small *Xba*I fragment from *dl*309 was mutagenized with nitrous acid and ligated to the untreated large fragment, and the plaques produced after transfection of the ligation mixture were screened for a host range (*hr*) phenotype. Such mutants grow normally on the Ad5-transformed cell line 293 (16) due to complementation by integrated copies of E1 DNA (1), but grow poorly on HeLa cells. The mutant described below (*hr*440) has a plaquing ratio (titer on cell line 293/titer on HeLa cells) of 10^4 and behaves as a conventional early mutant; i.e, viral DNA is not replicated in infected HeLa cells (data not shown).

E1A gene products. To facilitate discussion of the analysis of the mutant E1A products, the relevant structural information concerning wild-type E1A products is summarized in Fig. 1A. At early times during a wild-type infection of HeLa cells, E1A produces two coterminal but differently spliced mRNAs with lengths of 0.9 and 1.0 kilobases (kb), each of which is translated in vitro into two related polypeptides (Fig. 1A). The cause of this unusual protein heterogeneity has not been established, although the region responsible has been mapped to the downstream exon (28).

The initial characterization of *hr*440 (35) demonstrated that this mutant is defective in a splicing event required to generate the 0.9-kb mRNA. This defect was ascribed to one or both of two adjacent nucleotide changes which lie five and six nucleotides from the 5′ splice site for this mRNA (Fig. 1B). Furthermore, one of these changes introduces an amber codon into the reading frame used to translate the remaining 1.0-kb mRNA. As Fig. 2 shows, in vitro translation of E1A RNA purified from *hr*440-infected HeLa cells produced a single polypeptide with a molecular weight of 16,000, which was consistent with the length of 140 amino acids predicted for a premature termination product generated by the nonsense mutation. Therefore, *hr*440 displays both a transcriptional defect and a translational defect (Fig. 1A). It should be pointed out that an analysis of the *hr*440 DNA sequence corresponding to the C-terminal one-third of the 16K protein has revealed two additional mutations. However, one of these mutations is silent, and the other is conservative, converting a Glu codon to an Asp codon.

Transcriptional analysis of other early regions. To investigate the effects of the mutations on the

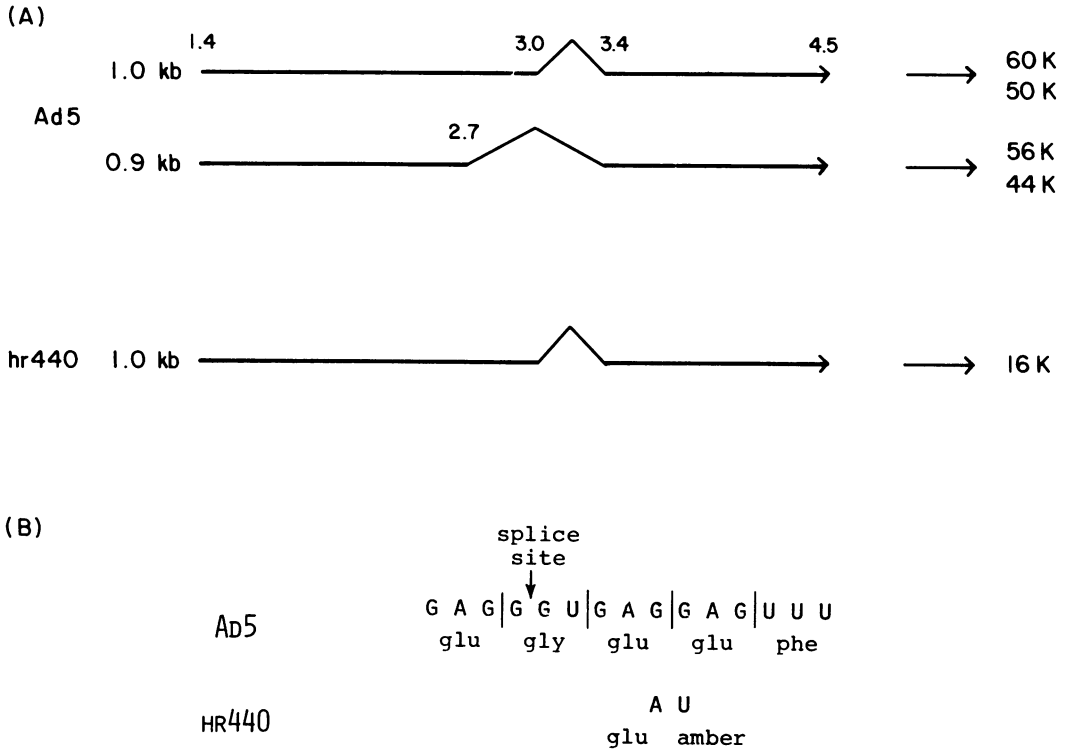

FIG. 1. Products of E1A in wild-type virus- and *hr*440-infected cells. (A) E1A transcripts and the proteins which they encode. The transcripts are represented by horizontal lines with arrows at the 3′ ends. The carets indicate sequences removed by splicing. The transcript lengths are noted on the left, and the molecular weights of the encoded proteins are noted on the right. The numbers along the transcripts indicate the conventional adenovirus map coordinates. The information concerning wild-type virus gene products was taken from references 5, 8, 10, 31, and 37. (B) Wild-type and mutant sequences near the 5′ splice site for the 0.9-kb mRNA. The arrow indicates the splice site for the 0.9-kb mRNA, and the vertical lines delineate the translational reading frame for the sequence in the 1.0-kb mRNA that extends beyond this site. The two adjacent mutations in *hr*440 are noted. The Ad5 sequence was taken from van Ormondt et al. (40).

expression of other regions of the genome, a transcriptional analysis was carried out by using the Berk-Sharp S1 procedure. Cytoplasmic RNA isolated early in infection of HeLa cells was hybridized separately to genomic DNA fragments from E1B, E2, E3, and E4, and the RNA-DNA hybrids were treated with S1 nuclease and displayed on a neutral agarose gel. Figure 3 shows that normal amounts of both E1B RNA and E4 RNA were produced by the mutant, whereas the levels of E2 RNA and E3 RNA were considerably lower in *hr*440-infected cells than in *dl*309-infected cells.

For unknown reasons, S1 analysis detects only the larger of the two major mRNAs from E1B (5). However, both of these mRNAs can be translated in vitro; a 55K protein is produced from the larger mRNA, and a 15K protein is produced from the smaller mRNA (10, 18, 27). SDS-polyacrylamide gel fractionation of the E1B translational products from mutant- and wild-type virus-infected cells (Fig. 4) demonstrated that *hr*440 produces both mRNAs in normal amounts.

The experiments described above were performed with cells infected at a multiplicity sufficiently low (5 to 10 PFU/cell) to avoid the multiplicity-dependent leakiness exhibited by many E1A mutants (19, 33). At this multiplicity, the yield from a single cycle of infection was 10^4-fold higher with cell line 293 cells than with HeLa cells. Furthermore, viral DNA replication was undetectable up to 29 h after infection of HeLa cells, and only trace amounts of replication were detected at a later time (40 h). At higher multiplicities (20 to 50 PFU/cell), *hr*440 exhibited a degree of leakiness similar to that reported for other E1A mutants. Finally, S1 analysis of HeLa cells infected with *hr*1 at a multiplicity of 5 to 10 PFU/cell substantiated the previous finding (3, 28) that expression of all of the major early regions is depressed in *hr*1-

infected cells (data not shown). Therefore, the unusual properties of *hr*440 are not a consequence of multiplicity-dependent leakiness.

Transformation. *hr*440 is unique among known E1A mutants in that it expresses E1B normally in the absence of normal expression of E1A. For this reason, *hr*440 permits an analysis of the separate contributions of these two transcriptional units to the viral transforming activity. Table 1 shows that *hr*440 is defective for transformation of baby rat kidney cells, whether the transforming agent is virus, full-length viral DNA, or a viral DNA fragment containing the transforming region. Furthermore, this defect has been mapped to a portion of the mutagenized *Xba*I restriction fragment (0 to 3.7 map units) that was used to generate *hr*440. The mapping was performed as follows. Two viral recombinants were constructed from restriction fragments derived from *hr*440 and *dl*309. One recombinant (*hr*A) contained *hr*440 sequences extending from the left end of the genome to an *Xma*I site at 2.8 map units; the remainder of the genome was derived from *dl*309. The other recombinant (*dl*A) contained *hr*440 sequences extending from the *Xma*I site to the *Xba*I site at map coordinate 3.7, and all other sequences came from *dl*309. The *hr*440 mutations responsible for the defects in E1A described above (Fig. 1B) are located at 2.7 map units and therefore are present only in the *hr*A recombinant. As expected, *hr*A remains defective for transformation, whereas *dl*A does not. Somewhat surprisingly, *dl*A transforms with a frequency greater than the wild type. This might be explained by the fact that *dl*A has a partial mutant phenotype (plaquing ratio, 70), probably due to one or more mutations downstream from the mutations shown in Fig. 1B. (A conservative change from arginine to lysine has been found at map coordinate 3.0 by DNA sequence analysis.) This phenotype may sufficiently reduce the cytopathic effect of the virus to allow a higher transformation efficiency, an explanation consistent with the observation that the transforming activity of *dl*A is directly correlated with the multiplicity of infection, whereas an inverse correlation is found when wild-type virus is used. Indeed, inspection of infected rat cells has indicated that the cytopathic effect of *dl*A is considerably less than that of wild-type virus.

To demonstrate directly that *hr*440 synthesizes normal amounts of E1B protein in the nonpermissive rat cells used for the transformation assays, E1B protein was assayed by immunoprecipitation with an adenovirus tumor antiserum. Lysates of baby rat kidney cells labeled from 18 to 24 h after infection were mixed with an antiserum (14B) that recognizes the 58K protein from E1B (26). (This protein corresponds to the 55K protein synthesized in vitro [38]). Figure 5 shows the immunoprecipitated proteins fractionated by electrophoresis through an SDS-polyacrylamide gel. Both *hr*440 and *hr*A produced levels of the 58K polypeptide that were comparable to those produced by *dl*309 and *dl*A, whereas the E1B protein was not detected in a control experiment with E1A deletion mutant *dl*312.

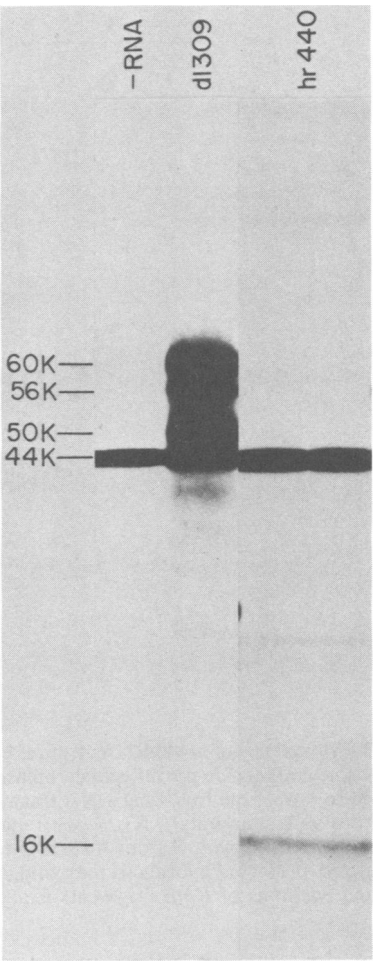

FIG. 2. E1A translational products from *hr*440-infected cells. A 200-μg sample of cytoplasmic RNA isolated early (9 h) in an infection of a suspension culture HeLa was hybridized to pHEB4 DNA immobilized on nitrocellulose filters. pHEB4 is a plasmid containing an Ad2 DNA fragment extending from the *Bal*I site at map coordinate 0.7 to the *Hpa*I site at map coordinate 4.3. The eluted RNA was translated in vitro, and the products were displayed on a 15% polyacrylamide gel containing SDS.

DISCUSSION

Regulatory behavior of E1A gene products. Like other E1A mutants, *hr*440 is defective in

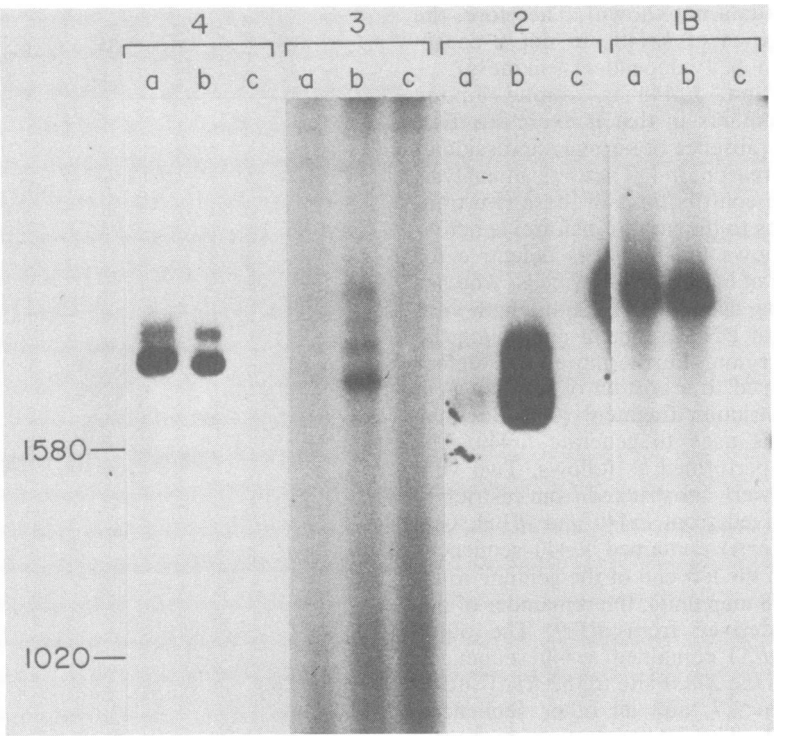

FIG. 3. S1 analysis of *hr*440 RNAs from E4, E3, E2, and E1B. HeLa cells were infected in the presence of 20 μg of cytosine arabinoside per ml, and cytoplasmic RNA was isolated 9 h later. A 200-μg sample of this RNA was hybridized to one of the following DNA fragments: E4, pAd20 (a plasmid containing *Hin*dIII fragment F from Ad2 [89.5 to 97.1 map units]); E3, *Sma*I fragment B from Ad5 (76.3 to 92.0 map units); E2, pRIB (a plasmid containing *Eco*RI fragment B from Ad2 [58.5 to 70.7 map units]); E1B, *Hpa*I fragment C from Ad5 (4.3 to 25.5 map units). S1-protected hybrids were fractionated on a neutral agarose gel. The markers on the left indicate the lengths and positions of *Hpa*I fragments E and F from Ad2. Lanes a, *hr*440; lanes b, *dl*309; lanes c, no RNA added.

TABLE 1. Transformation assays with baby rat kidney cells

Virus	Multiplicity (PFU/cell)	No. of foci[a]	DNA		Amt (μg/plate)	No. of foci[c]
			Intact	Fragment (0–15.8 map units)[b]		
Ad2	0.1	10	Ad5		5	13
	1	2	*hr*440		5	0
	15	0				
	50	0		Ad5	1	61
*hr*440	0.1	0		*hr*440	1	1
	1	0				
	15	0				
	50	0				
*hr*A	0.1	0				
	1	0				
	15	0				
	50	0				
*dl*A	0.1	3				
	1	56				
	15	>90[d]				
	50	>90[d]				

[a] Number of foci on three 6-cm plates.

[b] Because they were not resolved by preparative agarose gel electrophoresis, this assay used a mixture of *Xho*I-C (0 to 15.8 map units) and *Xho*I-B (83 to 100 map units).

[c] Number of foci on eight 6-cm plates.

[d] These values are approximate because the colonies were not well separated.

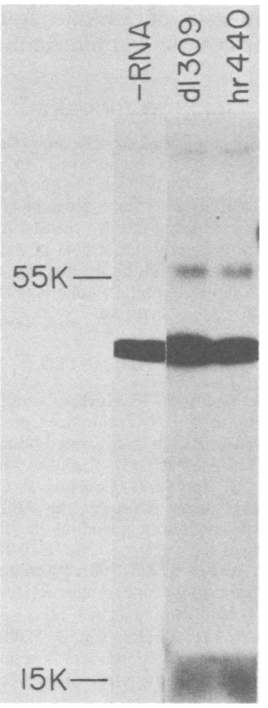

FIG. 4. E1B translational products from *hr*440-infected cells. E1B RNA from 200 μg of early cytoplasmic RNA was purified by hybridization to a *Hin*dIII-*Sma*I DNA fragment (7.7 to 10.8 map units) isolated from a plasmid (pAd6) containing *Hin*dIII fragment C (7.7 to 17.0 map units) from Ad2. The eluted RNA was translated in the presence of [^{35}S]methionine by using a reticulocyte lysate, and the translational products were displayed on a 15% polyacrylamide gel containing SDS.

the production of mRNAs from E2 and E3, but this mutant is unusual in that transcription from E1B and E4 is normal. A possible explanation for this finding is that the truncated E1A protein produced by *hr*440 retains the ability to induce the expression of some but not all of the viral early genes. The 16K polypeptide contains an amino acid sequence which is nearly identical to that encoded by the upstream exon for the 0.9-kb mRNA. By analogy with similar proposals for a number of cellular genes (14), this exon may encode a functional protein domain. Moreover, such a domain might maintain its activity even as a separate polypeptide. This postulate is consistent with the following evidence that the downstream exon for the 0.9- and 1.0-kb mRNAs is almost entirely expendable. Jones and Shenk (23) have isolated a frameshift deletion mutant (*dl*311) whose mutation maps 53 nucleotides downstream from the 3′ splice point common to the E1A mRNAs (33). Therefore, the E1A translational products from this mutant (10) should contain only 18 of the usual 105 amino acids encoded by the downstream exon. Notably, *dl*311 is a highly leaky mutant, having a plaquing ratio less than 1/1,000 that of most other E1A *hr* mutants, and it efficiently produces RNA from all of the conventional early regions (24). This suggests that E1A polypeptides containing primarily the amino acid sequences encoded by the upstream exons retain a considerable degree of activity. The 16K protein of *hr*440 may behave similarly. Why this behavior should be manifested by differential expression of the early regions is unclear. Perhaps E1A proteins normally have different affinities for early region regulatory sequences or proteins involved in early expression. The retention of partial activity by a truncated E1A protein might then result in substantial expression only from those regions whose normal expression involves a higher binding activity. It is particularly in-

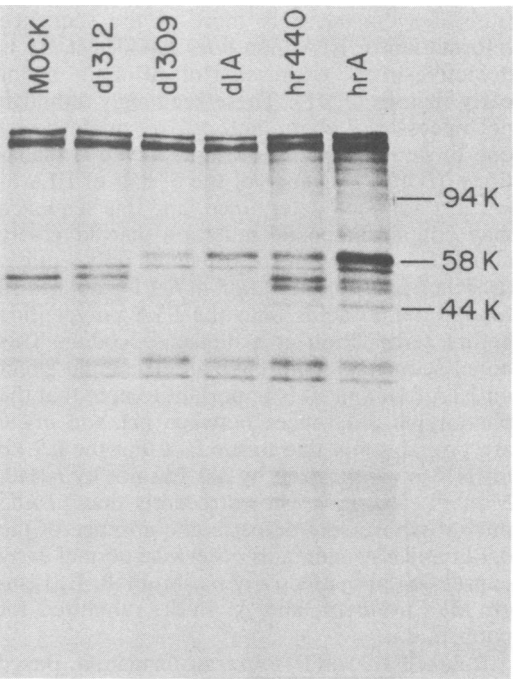

FIG. 5. E1B protein immunoprecipitated from infected baby rat kidney cells. Cell monolayers were labeled from 18 to 24 h after infection or mock infection, and proteins were immunoprecipitated by using a tumor serum (14B) that recognizes the 58K protein from E1B. Immunoprecipitated proteins were displayed on an SDS–15% polyacrylamide gel, along with marker proteins immunoprecipitated from a simian virus 40-transformed cell line (SV80) by using simian virus 40 tumor serum. The markers were simian virus 40 large T antigen (94K), a nonviral T antigen (58K), and actin (44K). To some extent, this reproduction falsely enhances the contrast between different band intensities. On the original autoradiogram, the bands corresponding to the E1B 58K protein showed a range of intensity of only two- to threefold.

triguing that the two regions whose expression is depressed during *hr*440 infection are transcribed from adjacent, divergent promoters; the 5′ ends of the mRNAs from E2 and E3 lie only 500 nucleotides apart (2, 2a, 13, 20). Perhaps normal E1A gene products enhance E2 and E3 expression in a concerted fashion.

Previous transcriptional studies of E1A mutants have concentrated on the following two isolates: a deletion mutant (*dl*312) missing the E1A promoter and most of the coding region (33), and a mutant (*hr*1) (19) suffering a single base deletion at map position 2.9 (32). The mutation in *hr*1 lies within the coding region of the 1.0-kb mRNA and in the intron for the 0.9-kb mRNA, so the normal 44K and 56K proteins are translated from the 0.9-kb mRNA, whereas the 1.0-kb mRNA encodes a polypeptide prematurely terminated at the nonsense codon introduced by the frameshift deletion (10). Therefore, *hr*1 translates considerably more of the sequence information in E1A than does *hr*440, yet *hr*1 is defective in the expression of all of the major early regions (3, 28). These seemingly although not necessarily contradictory data might be by one or both of the following resolved explanations. (i) The sequence of the 5′ half of E1A of *hr*1 has not been determined, and this sequence may contain a second mutation that adversely affects the activity of the truncated polypeptide. (ii) The frameshift mutation in *hr*1 introduces 10 foreign amino acids onto the E1A polypeptide before termination at a nonsense codon. This novel sequence could have a detrimental effect on E1A function. It is important to note that the phenotypic differences between *hr*1 and *hr*440 are probably not due to the fact that the 0.9-kb mRNA is synthesized by *hr*1 but not by *hr*440. Montell et al. have shown recently that an Ad5 mutant whose sole defect is the absence of the 0.9-kb mRNA maintains otherwise normal early expression and infectivity (C. Montell, E. Fisher, M. Caruthers, and A. Berk, submitted for publication).

Role of E1A and E1B in transformation. Based on their transcriptional study of *hr*1-infected cells, Berk et al. (3) proposed that the role of E1A in transformation may be to induce the expression of E1B. The results reported here indicate strongly that E1A serves another, more direct role in transformation. In support of this conclusion, Houweling et al. (21) found that a DNA fragment containing only E1A is sufficient to immortalize primary baby rat kidney cells, although the morphology of such transformants remains essentially fibroblastic. Furthermore, Shiroki et al. (34) have reported that the Ad5 mutant *dl*313, which does not contain E1B but maintains functional E1A (23), induces transformants with a similar morphology. The precise nature of the role of E1A in transformation awaits further genetic and biochemical studies.

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