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Expression of Viral DNA in Adenovirus Type 12-Transformed Cells, in Tumor Cells, and in Revertants

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The expression as cytoplasmic RNA of integrated human adenovirus type 12 (Ad12) DNA in transformed and tumor cell lines and in revertants was investigated. The transformed and tumor cells contained multiple copies of the viral genome, 3 to 22 copies per cell in different cell lines. The integrated Ad12 DNA molecules persisted intact or nearly intact and in most cases colinear with the virion DNA. In the revertant cell lines, which were derived from cell line T637 (22 copies of Ad12 DNA per cell), all of the Ad12 DNA molecules were lost (line F10) or only one copy and a fraction of a second copy persisted (line TR12). The size classes and map locations of Ad12-specific cytoplasmic RNAs in three Ad12-transformed hamster cell lines (T637, HA12/7, and A2497-3), in two revertant lines (F10 and TR12), in one Ad12-induced hamster (CLAC3), and in one rat brain tumor line (RBT12/3) were determined. Cytoplasmic RNA from uninfected B3 hamster cells and from human KB cells productively infected with Ad12 served as controls. In the latter control experiments, the RNA was isolated early or late postinfection. With respect to the amounts of Ad12-specific RNAs detected in cytoplasmic RNA from various Ad12-transformed or Ad12-induced tumor cell lines, we could not establish any correlations to the number of Ad12 genome copies integrated into the cellular DNAs. Thus, the expression of the integrated viral genomes in these lines was regulated by mechanisms more complicated than simple gene dosage effects. Using cloned fragments of Ad12 DNA as hybridization probes, we analyzed the cytoplasmic RNAs from the cell lines mentioned by electrophoresis on agarose gels, blotting, and DNA-RNA hybridization. For each transformed and tumor cell line, except for the revertants, several size classes of Ad12-specific cytoplasmic RNA were detected for the early E1, E2, and E4 regions of Ad12 DNA. Some of these size classes were similar but not identical to those observed in cytoplasmic RNA isolated early from human KB cells productively infected with Ad12. Only cell lines A2497-3, T637, and RBT12/3 contained several size classes of cytoplasmic RNA homologous to the E3 region of Ad12 DNA. Weak homologies to the E1 region of Ad12 DNA were also detected in the revertant lines F10 and TR12. Late regions of Ad12 DNA were expressed as cytoplasmic RNA in cell lines CLAC3 and RBT12/3. Weak homologies were detected between certain segments of the Ad12 genome (the *Eco*RI-B, -C, and -D fragments) and the cytoplasmic RNA from uninfected hamster cells. These homologies had no apparent counterpart at the level of DNA, perhaps because these homologies could be detected only due to an overrepresentation of RNA sequences. In preliminary experiments, we failed to detect the expression as cytoplasmic RNA of the so-called virus-associated RNA in transformed and tumor cell lines. Virus-associated RNA represents a population of low-molecular-weight RNAs that map at around 30 fractional length units on the viral genome.

The patterns of integration and persistence of viral DNA in adenovirus type 12 (Ad12)-transformed hamster cells and of Ad12-induced hamster and rat tumor cells have been investigated in detail (8, 9, 13, 22, 26; I. Kuhlmann and W. Doerfler, submitted for publication). Although each transformed and tumor line appears to have its specific properties, certain general features emerge with respect to the integration patterns of Ad12 DNA. Multiple copies of the Ad12 genome are integrated in complex arrangements. A portion of the Ad12 DNA molecules is inserted colinearly with virion DNA. However, within sequences close to the termini of the Ad12 DNA, selective amplifications have been observed (22). Moreover, the persistence of Ad12

DNA in virus-induced hamster tumors is characterized by the relative underrepresentation of the terminal segments of Ad12 DNA. This underrepresentation may merely reflect the random distribution of the sites of integration of the Ad12 genome (Kuhlmann and Doerfler, submitted for publication). At present, we do not understand the mechanisms involved in viral DNA integration. As a working hypothesis, we favor the view that early in the interactions of the virus and host genomes, one or a few copies of Ad12 DNA are inserted intact and colinear with the virion DNA. Subsequently, the viral DNA and adjacent cellular sequences are amplified. Deletions and rearrangements observed may occur during or may be somehow related to the amplification events.

Limited information is available on the expression of the adenovirus genome in transformed and tumor cells. In some of the Ad12-transformed hamster cell lines, only early genes of Ad12 are transcribed into mRNA (16, 18). In addition to early genes, some of the late genes appear also to be transcribed into mRNA in Ad12-induced rat brain tumor cells (13). Virus-specific mRNA has been mapped in a number of Ad2-transformed rat cells (20) and in rat cells transformed by the left end of Ad7 DNA (31).

In a number of viral systems (4, 5, 12, 24, 25, 29), there is evidence for an inverse relationship between the extent of DNA methylation at the 5′-CCGG-3′ sites and the level of expression into mRNA in specific segments of the viral DNA (24, 25, 29). The possible role of specific patterns of DNA methylation for gene regulation in eucaryotic systems has promoted a great deal of research in this field (Doerfler, J. Gen. Virol., in press). However, we are far from understanding at a molecular level the significance of 5-methylcytosine as a possible regulatory signal. Viral systems provide, however, a resourceful tool for detailed studies on this topic.

In the present study, we analyzed by blotting and DNA-RNA hybridization techniques the size classes of Ad12-specific cytoplasmic RNAs in a number of Ad12-transformed hamster cell lines and of Ad12-induced hamster and rat tumor lines. The different size classes of Ad12-specific RNA were mapped on the viral genome. Evidence is adduced for the existence of homologies between certain segments of the Ad12 genome and cytoplasmic RNA from uninfected hamster cells.

MATERIALS AND METHODS

Cells and virus. Lines T637, HA12/7, and A2497-3 were derived from hamster cells by transformation with Ad12 in culture (26). Line CLAC3 (22) represents cells from an Ad12-induced hamster tumor; these cells were carried in passage 30 to 40 in culture. Line RBT12/3 was established from an Ad12-induced rat brain tumor (13). The morphological revertants F10 and TR12 arose spontaneously in cultures of T637 cells and were characterized earlier (9–11). All of these cell lines were maintained in culture in Dulbecco modified Eagle medium (1) reinforced with 5% fetal calf serum. Cells were passaged twice weekly.

The B3 subline of BHK-21 cells was carried in Dulbecco medium containing 10% horse serum. Human KB cells were maintained in Eagle medium with 10% newborn calf serum. Human Ad12 (Huie strain) was propagated on KB cells in monolayer or suspension cultures. The virus was purified as described previously (6). In some experiments, cytoplasmic RNAs were extracted and analyzed as controls from Ad12-infected KB cells at 8 to 10 h postinfection (early RNA) or at 45 to 48 h postinfection (late RNA). It was shown previously that Ad12 DNA replication in KB cells starts at 12 to 14 h postinfection (19).

Viral DNA and cloned viral DNA fragments. Ad12 DNA was prepared as described elsewhere (7). For all experiments, molecular clones of fragments of Ad12 DNA (S. Vogel, M. Brötz, I. Kruczek, R. Neumann, U. Winterhoff, D. Eick, and W. Doerfler, Gene, in press) were used as hybridization probes. The fragments *Bam*HI-B and -D were cloned in pBR322 DNA (3), fragment *Eco*RI-B was cloned in λgtWES·λB′ DNA (27), and fragments *Eco*RI-A* and -D were cloned in pBR325 DNA (2). Fragment *Eco*RI-A* comprised about 5 kilobase pairs (kb) from the right-terminal Ad12 DNA (Vogel et al., in press). The locations of individual DNA fragments on the Ad12 DNA map are indicated in Fig. 2. The left terminal *Eco*RI-C fragment (5.4 kb) of Ad12 DNA was cloned in plasmid pBR322 and was a gift of A. van der Eb, University of Leiden, Leiden, Holland.

Nick translation of DNA. Ad12 DNA or molecular clones of Ad12 DNA fragments were labeled with [α-^{32}P]CTP and [α-^{32}P]TTP by the nick translation procedure (17) as outlined elsewhere (28).

Preparation of cytoplasmic RNA from infected or transformed cells. Cells growing in monolayer cultures were washed twice with ice-cold Tris-saline; the plastic dishes containing the cells were placed on crushed ice before the medium was removed. The cells were then scraped off and suspended in 0.01 M NaCl–0.01 M Tris-hydrochloride, pH 8.5–0.005 M $MgCl_2$. The cells were pelleted by low-speed centrifugation, suspended in 1 ml of 0.01 M NaCl–0.01 M Tris-hydrochloride, pH 8.5–0.0015 M $MgCl_2$–0.5% Nonidet P-40–0.05% deoxycholate per 2.5×10^7 cells, and allowed to disintegrate on ice for 5 to 7 min. Subsequently, the nuclei were pelleted by low-speed centrifugation, and the supernatant was taken off and recentrifuged. An equal volume of 0.1 M Tris-hydrochloride, pH 8.0–0.3 M NaCl–0.015 M EDTA was added. The RNA was further purified by making the solution 1% in sodium dodecyl sulfate and by three to five extractions with equal volumes of phenol and chloroform. The phenol-chloroform mixture was saturated three times with 0.1 M sodium acetate, pH 5.2. Chloroform was mixed with isoamyl alcohol at a ratio of 24:1. Finally, the RNA was ethanol precipitated (two to three volumes of ethanol) and stored at −20°C. Before use, all buffers

were treated with small amounts of diethyl pyrocarbonate and were subsequently autoclaved.

Agarose gel electrophoresis of RNA. Cytoplasmic RNA was analyzed in horizontal 0.67 to 1.5% agarose slab gels in MOPS buffer (0.02 M morpholinopropanesulfonic acid–5 mM sodium acetate–1 mM EDTA, pH 7.0). After melting the agarose and cooling the solution to 60°C, one-sixth volume of 37% formaldehyde was added, and the gels were then poured. Samples were suspended in 50% formamide and 2.2 M formaldehyde in MOPS buffer containing about 1% Ficoll and 0.2% bromophenol blue. Samples were heated to 60°C for at least 5 to 10 min immediately before being applied to the gel slots. Samples of 10 μg of RNA per slot were used and were electrophoresed at 50 V for 14 to 20 h at room temperature. In some experiments, samples were electrophoresed at 150 to 180 V for 5 to 6 h at room temperature, and these conditions seemed to improve resolution. As molecular weight markers, Ad12 DNA cleaved with the restriction endonuclease *Hin*dIII was coelectrophoresed on all gels. The sample buffer described above was used for Ad12 DNA fragments as well. The Ad12 DNA fragments were denatured by this treatment. The denatured *Hin*dIII fragments of Ad12 DNA had sizes of 5.4_5 (A fragment), 4.7_9 (B), 3.2_4 (C through E), 2.3_9 (F and G), 1.7_2 (H), 1.4_2 (I), and 1.1_2 (J) kb, respectively.

Transfer of RNA from gels to nitrocellulose filters. After electrophoresis, the RNA was transferred to nitrocellulose filters (BA85; Schleicher & Schuell Co., Dassel, West Germany) according to the Southern technique (21). The gels were not pretreated; nitrocellulose filters were prewetted in 1.5 M NaCl–0.15 M sodium citrate (10× SSC), and the same solution was used for transfers that lasted 36 to 40 h.

DNA-RNA hybridization and autoradiography. ^{32}P-labeled Ad12 DNA or cloned restriction endonuclease fragments were used as probes in hybridization experiments. Experimental conditions are described elsewhere (30). We modified the wash procedure slightly. After the hybridization period (16 to 24 h), filters were washed three times for 5 min at room temperature in 300 ml each of 2× SSC and 0.1% sodium dodecyl sulfate and then three times for 2 h at 50°C in 300 ml of 0.1× SSC and 0.1% sodium dodecyl sulfate. After the filters were dried at 80°C, they were autoradiographed at −80°C on Kodak XR-5 film using intensifier screens.

RESULTS

Survey of cell lines investigated for Ad12-specific sequences in cytoplasmic RNA. Integration patterns of viral DNA have been determined for a large number of Ad12-transformed cell lines or Ad12-induced tumors and tumor cell lines. Representative cell lines were selected for the analyses of Ad12-specific cytoplasmic RNAs. The hamster cell line T637 has been obtained by transformation of BHK-21 cells (subline B3) with Ad12 (23). The morphological revertants F10 and TR12 have been isolated from line T637 without pretreatment (10, 11). The revertant line F10 has lost all Ad12 DNA sequences; the revertant line TR12 has lost all but one copy and a fragment of a second copy of Ad12 DNA (9). Cell lines HA12/7 (32) and A2497-3 (A. M. Lewis, Jr., personal communication) have been derived from primary hamster cells by transformation with Ad12 in culture. Lines CLAC3 (22) and RBT12/3 (13) have been established from an Ad12-induced subcutaneous hamster tumor and an Ad12-induced rat brain tumor, respectively. The hamster cell line B3 was used as a control in all experiments. In some experiments, RNAs from human KB cells productively infected with Ad12 were isolated early or late postinfection and were used as positive controls.

Ad12-specific cytoplasmic RNA in Ad12-transformed lines and in Ad12-induced tumor cell lines. Cytoplasmic RNA was isolated from transformed, tumor, or control cells and was analyzed by gel electrophoresis, blotting and hybridization to Ad12 DNA or selected Ad12 DNA fragments. All DNA fragments used were available as molecular clones in plasmid pBR322 DNA, in plasmid pBR325, or in bacteriophage λgtWES·λB′ DNA (Vogel et al., in press). The data presented in Fig. 1 demonstrate that very similar size classes of Ad12-specific cytoplasmic RNA could be detected in lines CLAC3, A2497-3, HA12/7, and RBT12/3. Similar results were obtained with cytoplasmic RNA from line T637 (data not shown). Minor differences in the distribution of RNA in different size classes existed. The total amount of Ad12-specific RNA sequences varied from cell line to cell line when the intensities of autoradiographic blackening of the films were taken as an indirect measure. Apart from low-molecular-weight material, no homologies were detectable between Ad12 DNA and the RNAs of the revertant lines or of B3 control cells.

A more detailed analysis of the expression of Ad12 genes in transformed cells became feasible with the availability of cloned fragments of Ad12 DNA (Vogel et al., in press). It could be demonstrated by blot analyses that the plasmid vectors (pBR325 and hence pBR322) did not have any homologies to cytoplasmic RNAs from the cell lines investigated (data not shown). Thus, cloned fragments of Ad12 DNA could be used as highly specific probes in hybridization experiments. Cytoplasmic RNAs from cell lines as indicated (Fig. 2) were fractionated on horizontal agarose slab gels, blotted onto nitrocellulose filters, and hybridized to the following cloned restriction endonuclease fragments which were labeled with ^{32}P by nick translation: *Eco*RI-C (Fig. 2A), *Eco*RI-D (Fig. 2B), *Eco*RI-B (Fig. 2C), *Bam*HI-C (Fig. 2D), *Bam*HI-B (Fig. 2E), and *Eco*RI-A* (Fig. 2F). The locations of these frag-

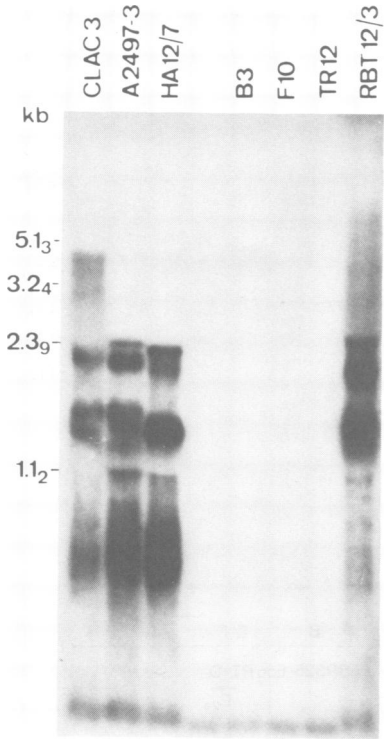
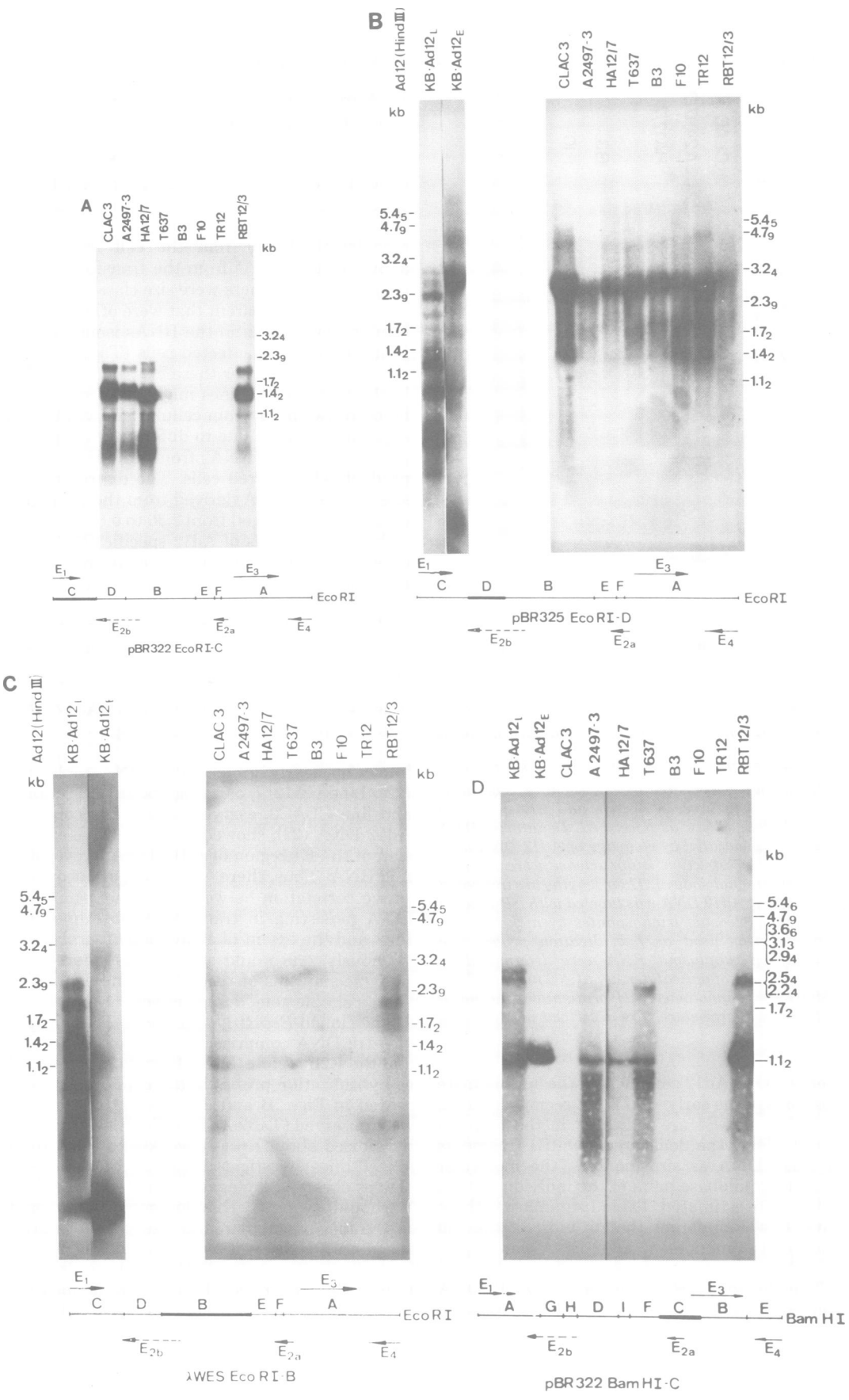


FIG. 1. *Size distribution of Ad12-specific cytoplasmic RNAs in Ad12-transformed hamster cells (A2497-3 and HA12/7), in revertants (F10 and TR12), and in Ad12-induced tumor lines (CLAC3 and RBT12/3). Cytoplasmic RNAs (10 μg/slot) from cell lines as indicated were separated by electrophoresis on horizontal 0.67% agarose slab gels and transferred to nitrocellulose filters as described. Denatured DNA fragments generated by cleavage of Ad12 DNA with the HindIII restriction endonuclease were used as size markers (not shown). Their lengths are indicated in kilobases. Ad12 DNA was labeled with ^{32}P-labeled deoxyribonucleoside triphosphates by nick translation and was used as hybridization probe. The amounts of cytoplasmic RNA were determined by measurements of optical density at 260 nm in a Zeiss PMQII spectrophotometer. Hybridization and autoradiographic procedures are described in Materials and Methods.*

ments on the Ad12 genome and the approximate positions of the early viral DNA regions E1, E2a, E2b, E3, and E4 are indicated in the maps in Fig. 2. Using the denatured *Hin*dIII fragments of Ad12 DNA as size markers, the molecular weights in kilobase pairs of the individual RNA bands were estimated. Table 1 summarizes these data. It was apparent that in most of the cell lines, early functions of Ad12 DNA were represented among the cytoplasmic RNA molecules.

The cloned *Eco*RI-C fragment of Ad12 DNA yielded very strong signals with cytoplasmic RNA from cell lines CLAC3, A2497-3, HA12/7, and RBT12/3; weak hybridization was observed with cytoplasmic RNA from line T637, and none was observed with cytoplasmic RNA from the control line B3 and the revertant lines F10 and TR12 (Fig. 2A). Table 1 shows the individual size classes of Ad12-specific RNA discernible in cytoplasmic RNA from the cell lines listed above. In the RNAs from the transformed and tumor cell lines, there were size classes of Ad12-specific RNAs apparent that were of higher molecular weight than in the RNAs isolated early from productively infected cells (Table 1). It is not yet clear whether these classes of E1 RNA in the transformed lines might have originated by cotranscription from cellular and viral DNA sequences or were due to differences in splicing patterns between RNAs from transformed and productively infected cells. The distribution of size classes of RNA derived from the E1 region of Ad12 DNA ranged from 2.36 to 0.65 kb. Since rather sharp bands of Ad12-specific RNA were observed, we considered it unlikely that any of these size classes of RNA constituted degradation products. The Ad12-specific sequences of 2.4 kb in lines B3, F10, and TR12 (Table 1) were not apparent in Fig. 2A but were seen as weak hybridization signals in experiments not shown here. One additional feature apparent in Fig. 2A deserved comment: the E1 region of Ad12 DNA was very poorly expressed in cytoplasmic RNA of line T637, which contains about 22 copies of intact or nearly intact Ad12 DNA molecules (22). Line HA12/7, carrying about three copies, and line CLAC3, carrying about five copies of Ad12 DNA (22), showed very extensive expression of the E1 region of Ad12 DNA in cytoplasmic RNA. Thus, there was no apparent quantitative correlation between the amount of viral DNA persisting in transformed or tumor cell lines and the extent of expression of viral DNA. Obviously, one would expect other more complex mechanisms of regulating gene expression than a simple gene dosage effect.

The cloned *Eco*RI-D and *Eco*RI-B fragments of Ad12 DNA comprised the major late regions and the E2b region. These fragments were used as hybridization probes in the experiments presented in Fig. 2B and 2C. Several size classes were observed (Table 1). The *Eco*RI-D fragment hybridized about equally weakly to RNA from all cell lines investigated (Fig. 2C), and the size classes observed are again tabulated in Table 1. It should be noted that low-molecular-weight RNA, in particular in the size class of Ad12-specific VA RNA, was not observed (Fig. 2B, C). The *Eco*RI-B and *Eco*RI-D fragments also contained the early region E2b, which mapped between about 16 and 34 units on the Ad12 ge-


A
CLAC3
A2497-3
HA12/7
T637
B3
F10
TR12
RBT12/3
kb
3.24
2.39
1.72
1.42
1.12
E1
C
D
B
E F
A
E3
EcoRI
E2b
E2a
E4
pBR322 EcoRI-C
B
Ad12 (HindIII)
KB-Ad12L
KB-Ad12E
5.45
4.79
3.24
2.39
1.72
1.42
1.12
pBR325 EcoRI-D
C
Ad12(HindIII)
KB Ad12I
KB Ad12t
λWES EcoRI-B
D
KB Ad12I
KB Ad12E
5.46
4.79
3.66
3.13
2.94
2.54
2.24
1.72
1.12
A
G H
D
I
F
C
B
E
Bam HI
pBR322 BamHI-C

nome. Thus, part of the hybridization obtained with these fragments might have been due to the expression of the E2b region. In addition, there seemed to exist some non-Ad12-specific hybridization which will be commented on below.

The E2a region of Ad12 DNA is located on the *Bam*HI-C fragment, which was used for hybridization experiments presented in Fig. 2D and Table 1. The same fragment would also hybridize to RNA derived from the E3 region of Ad12. In the experiment described in Fig. 2D, cytoplasmic RNA from line CLAC3 did not anneal to the *Bam*HI-C fragment. In other experiments (not shown), very weak bands were observed (Table 1).

The E3 region straddling the *Bam*HI-C and *Bam*HI-B fragments was found to be expressed as cytoplasmic RNA in cell lines A2497-3, T637, and RBT12/3; the other cell lines did not contain cytoplasmic RNA corresponding to this segment of Ad12 DNA (Fig. 2E). It has been reported previously that in line HA12/7 the E3 region is not expressed as mRNA (16). Very strong signals were, however, observed in lines A2497-3 and RBT12/3 which corresponded to rather precisely the same size classes in all cell lines, including the two size classes detectable in cytoplasmic RNA from line T637 (Fig. 2E). The *Bam*HI-C and *Bam*HI-B fragments exhibited no homology to cytoplasmic RNA from the B3 control line or from the two revertants analyzed.

When the right-terminal *Eco*RI-A* fragment of Ad12 DNA (Vogel et al., in press) that contained the entire E4 and part of the E3 region of Ad12 DNA was used as the hybridization probe, considerable differences in the Ad12-specific size classes were observed in cytoplasmic RNA from the different cell lines (Fig. 2F, Table 1), although each band occurring in one cell line had a counterpart of apparently identical size in the cytoplasmic RNA of at least one other cell line. The cytoplasmic RNA isolated from line B3, F10, or TR12 showed no homology to the *Eco*RI-A* fragment (Fig. 2F).

Although it appears obvious, it should be stated here with respect to all size class comparisons in this report that similarities or even

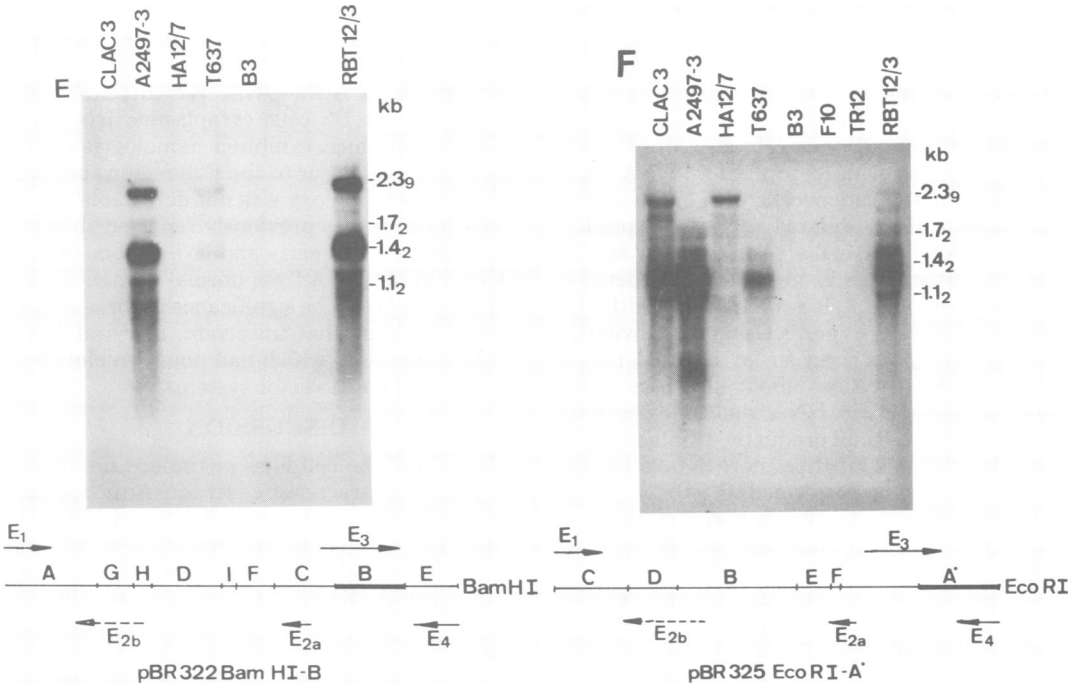


FIG. 2. *Analyses of cytoplasmic RNAs from Ad12-transformed lines, from revertants, from Ad12-induced tumor lines, from B3 cells, and from KB cells productively infected with Ad12 using cloned fragments of Ad12 DNA as hybridization probes. Experimental conditions were similar to those described in Fig. 1 and in Materials and Methods. The locations of the fragment probes used in each set of experiments are indicated by closed bars in the maps in each figure. The vectors used are also designated. In panels B and C, analyses of cytoplasmic RNAs isolated from KB cells productively infected with Ad12 at early (KB·Ad12$_E$, 8 to 10 h) and late (KB·Ad12$_L$, 45 to 48 h) times postinfection were also included. The size scales (in kilobases) refer to the locations of the single-stranded HindIII fragments of Ad12 DNA.*

TABLE 1. *Summary of hybridization data*[a]

Cell line[b]	Ad12-specific RNA size classes (kb) with homologies to the following segments of Ad12 DNA					
	*Eco*RI-C (E1)	*Eco*RI-D (E2b + L)	*Eco*RI-B (E2b + L)	*Bam*HI-C (E2a)	*Bam*HI-B (E3)	*Eco*RI-A* (E3 + E4)
KB·Ad12$_E$	1.4$_7$, 0.6$_7$		3.3$_3$, 1.2$_1$, 0.2$_6$	2.1$_5$, 0.9$_7$	2.0$_5$, 1.2	2.7$_5$, 2.6$_9$, 1.5$_7$, 1.3$_7$
KB·Ad12$_L$	0.9$_4$, 0.7$_1$, 0.4$_1$		2.4$_2$, 2.0, 1.3$_3$, 1.1$_8$ 0.8$_8$, 0.5$_2$, 0.2$_6$	2.4$_5$, 2.2$_7$, 0.9$_1$	2.5$_5$, 1.1$_5$	1.3$_6$, 0.6$_8$
CLAC3	2.1$_5$, 1.6$_7$–1.4$_2$, 1.1$_1$, 0.7$_1$–0.6$_7$	4.4$_8$, 2.7$_9$	2.7$_3$, 1.1$_8$, 0.6$_4$	(2.3$_6$, 1.0$_9$)		2.3, 2.0$_1$, 1.6$_7$, 1.2$_7$, 1.0$_6$
A2497-3	2.1$_2$, 1.6$_1$–1.4$_2$, 1.1$_2$, 0.7$_2$–0.6$_8$	2.7$_3$	2.7$_3$, 1.2$_1$	2.2$_7$, 1.0$_5$	2.0$_9$, 1.7$_6$, 1.4, 1.1$_7$, 0.9$_7$	1.7, 1.4$_7$, 1.2$_4$, 1.1$_8$
HA12/7	2.2$_2$, 2.0$_5$, 1.5–1.3$_3$, 1.1, 0.8, 0.69–0.6$_5$	4.1$_8$, 2.7$_3$	2.8$_8$, 1.2$_7$	2.3$_3$, 1.0$_6$		3.0 (1.9$_4$, 1.7, 1.0$_3$)
T637	2.3$_6$, 2.0, 1.4$_5$–1.3$_6$	4.1$_8$, 2.7$_6$, 1.9$_4$	2.8$_2$, 1.2$_7$	2.3$_5$, 1.0$_6$	2.0$_9$, 1.7$_4$, 1.3$_6$, 1.1$_9$	(1.7, 1.4$_5$, 1.2$_4$, 1.0$_6$)
B3	(2.4$_2$)	4.1$_8$, 2.7$_5$, 2.0	2.9$_7$, 1.2$_6$			
F10	(2.4$_2$)	2.7$_3$, 1.9$_7$	3.0$_4$, 1.3			
TR12	(2.4$_2$)	4.4$_8$, 2.7$_3$, 1.9	2.8$_2$, 1.3			
RBT12/3	1.9$_9$, 1.5$_1$–1.3$_5$, 1.0$_5$, 0.6$_8$–0.6$_5$	2.8$_2$	2.6$_7$, 2.2$_1$, 1.2$_7$, 0.6$_7$	2.5$_3$, 1.1	2.5, 2.1$_5$, 1.8$_5$, 1.4$_5$, 1.2, 1.0$_2$	2.4, 2.1$_1$, 1.8$_5$, 1.7$_5$, 1.4$_7$, 1.2$_7$, 1.0$_8$

[a] Summary of the results presented in Fig. 2. Most of the data obtained with RNA from productively infected cells were not included in Fig. 1 and 2. Numbers in parentheses indicate very weak bands in the autoradiograms. The locations of restriction endonuclease fragments as indicated are shown in Fig. 2. The subscripts represent the second digit after the decimal point; subscripts have been used to indicate that these latter figures are approximations. The smallest *Hin*dIII marker fragment used was 1.1$_2$ kb, hence all values listed below that size class could be outside the linear range of the gel.

[b] KB·Ad12$_E$ and KB·Ad12$_L$ refer to KB cells productively infected with Ad12. The cytoplasmic RNA was isolated early (8 to 10 h, KB·Ad12$_E$) or late (45 to 48 h, KB·Ad12$_L$) after infection.

identities in size of viral RNA products observed in specific segments of the Ad12 genome do not prove sequence identities. With this sensible reservation in mind, one might also compare size classes observed in cytoplasmic RNA from transformed and tumor cells with those seen in cytoplasmic RNA isolated early from productively infected KB cells (Table 1). In several instances there was good congruence between the two sets of size class tabulations, although there was rarely a perfect match. Obviously, with the methods used so far it could not be expected that decisions about identities of different Ad12-specific RNA molecules isolated from transformed and productively infected cells could be reached. Further, more detailed analyses will have to be performed.

Homologies between certain regions of Ad12 DNA and cytoplasmic RNA from B3 cells. The data presented in Fig. 2B and 2C demonstrated that homologies existed between the *Eco*RI-B and -D fragments of Ad12 DNA and cytoplasmic RNA from B3 cells. It could also be shown that the amount of DNA hybridized at 50°C was considerably less than that at 42°C (data not presented). Cytoplasmic RNA from primary hamster muscle cells did not exhibit any homology to the *Eco*RI-D fragment (data not shown). We also tested for a corresponding homology by DNA-DNA hybridization using the Southern blotting technique (21). There was no evidence for hybridization between DNA from B3 cells and the cloned *Eco*RI-D fragment of Ad12 DNA. We therefore concluded that in B3 cells cytoplasmic RNA was synthesized which exhibited homologies to the major late regions or to the E2b regions of Ad12 DNA. This homology was not detectable at the DNA level. It was previously reported that homologies between early and late regions of Ad2 DNA and the RNA from normal human tissues were found (14). The significance of these results had been somewhat uncertain, for viral DNA probes were used which had not been cloned in any of the known vector systems.

DISCUSSION

In each of the cell lines or tumor lines investigated, a characteristic set of Ad12-specific RNA molecules is synthesized. These RNA molecules are derived mainly from the early segments of the Ad12 genome; in some cell lines (CLAC3 and RBT12/3), cytoplasmic RNA is also derived from late segments (13). The data compiled in Table 1 summarize all of the results obtained and list the size classes of Ad12-specific RNAs identified in the cytoplasmic RNA of each cell line. For comparison, the size classes in cytoplasmic RNA isolated from productively infected cells both early and late postinfection have also been tabulated (Table 1). It has not yet been determined whether an increased size of RNA molecules in transformed or tumor cells as compared with productively infected cells

suggests that these RNAs are cotranscripts derived from both cellular and integrated viral DNA sequences.

Aside from homologies to the *Eco*RI-B and -D fragments of Ad12 DNA (Fig. 2B, C), which are also found in BHK-2/(B3) cells, cytoplasmic RNA from the revertant lines F10 and TR12 does not contain virus-specific RNA molecules. These data confirm earlier findings (9) indicating that the persisting Ad12 DNA sequences in revertant cell lines are not expressed extensively as cytoplasmic RNA.

The interpretation of the data on the expression of late regions of the Ad12 genome in transformed and tumor cell lines is complicated by the finding of homologies between the cytoplasmic RNA from B3 hamster cells and the *Eco*RI-B and -D fragments. The extent of hybridization between these Ad12 DNA segments and cytoplasmic RNA from B3 hamster cells and the hamster lines investigated with the exception of lines CLAC3 and RBT12/3 is markedly decreased when the temperature used in the annealing experiments is raised from 42 to 50°C. Weak homologies are still observed at the higher temperature. Corresponding homologies have not been detected by DNA-DNA hybridization. We conclude that hamster cells produce cytoplasmic RNA sequences with weak sequence homologies to the *Eco*RI-B and -D fragments of Ad12 DNA. The paradox of apparent homologies between cytoplasmic RNA from control B3 cells and sequences in the *Eco*RI-B and *Eco*RI-D fragments of Ad12 DNA and the failure to detect corresponding homologies at the DNA level might be explained by one or both of the following possibilities: (i) at the DNA level, these homologies might be restricted to very short sequences and might not register in DNA-DNA hybridization, which is known to be less efficient than DNA-RNA hybridization; (ii) these homologies might be detectable in DNA-RNA hybridizations, because the corresponding RNA molecules might be markedly overproduced even in uninfected control cells, whereas at the DNA level these sequences might be represented once per cell. In any event, the nature of these sequences has not yet been elucidated.

Homologies between certain segments of the Ad2 genome and RNA from a variety of normal human tissues have been reported previously (14). In contrast to our studies, however, these authors have not reported using viral DNA fragments that had been cloned in suitable plasmid or bacteriophage vectors. As a note of caution, we should like to mention the results of experiments (M. Westphal and W. Doerfler, unpublished data) in which apparent homologies between Ad2 DNA and KB cell DNA were observed. These homologies decreased when the Ad2 virions used for DNA extraction were treated with DNase and were repurified before the viral DNA was extracted. Moreover, cloned Ad2 DNA fragments did not reveal homologies to KB cell DNA (15). We therefore conclude that observations of apparent homologies between viral and cell DNA may be misleading when these homologies are derived from experiments in which uncloned viral DNA has been used.

In cell lines CLAC3 and RBT12/3, several distinct size classes of Ad12-specific cytoplasmic RNA are detectable which hybridize to the *Eco*RI-D, the *Eco*RI-B, or the *Bam*HI-D fragment. These RNA molecules are clearly different from the weak homologies described. Their existence provides evidence for the expression of late viral DNA segments in Ad12-induced hamster and rat tumor lines and confirms earlier results (13). The *Eco*RI-B and -D fragments of Ad12 DNA also harbor the early region E2B, which has not been precisely mapped. Preliminary results (H. Esche, unpublished data) suggest this region to be located between approximately 16 and 34 map units on the Ad12 genome. Thus, cytoplasmic RNA hybridizing to the *Eco*RI-B and -D fragments might be derived from the major late regions or the early region E2b. It had been shown earlier (13) that cytoplasmic RNA from cell line RBT12/3 hybridizes to the exclusively late fragment *Bam*HI-D, and data not shown here indicate that cytoplasmic RNA from cell line CLAC3 also hybridizes to this fragment. Thus, cell lines RBT12/3 and CLAC3 seem to express late segments of the persisting Ad12 genome.

Virus-associated RNAs comprise a set of low-molecular-weight RNA molecules which are synthesized in productively infected cells and which map at around 30 fractional length units on the viral genome. Preliminary results suggested that Ad12-specific virus-associated RNA was not detectable in the cytoplasmic RNA extracted from cell lines T637, HA12/7, A2497-3, TR7, TR12, and B3. Ad12-specific virus-associated RNA was, however, found in cytoplasmic RNA from KB cells productively infected with Ad12 at both early and late times postinfection.

It was reported previously that the levels of DNA methylation at the 5′-CCGG-3′ sites in the integrated Ad12 genomes persisting in revertants of cell line T637 are very high (9). An inverse correlation between the extent of adenovirus DNA methylation of integrated viral genomes and the level of expression of specific segments has been established (24, 25, 29). The absence of Ad12-specific cytoplasmic RNA sequences in revertants of line T637 therefore pro-

vides a further example of this inverse correlation, which has since been confirmed in several other viral systems (Doerfler, J. Gen. Virol., in press). It remains unknown in what way the signal of 5-methylcytosine might influence gene regulation.

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LITERATURE CITED

1. **Bablanian, R., H. J. Eggers, and I. Tamm.** 1965. Studies on the mechanism of poliovirus-induced damage. I. The relation between poliovirus-induced metabolic and morphological alterations in cultured cells. Virology **26:** 100-113.
2. **Bolivar, F.** 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique EcoRI sites for selection of EcoRI generated recombinant DNA molecules. Gene **4:** 121-136.
3. **Bolivar, F., R. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles: II. A multipurpose cloning system. Gene **2:**95-113.
4. **Cohen, J. C.** 1980. Methylation of milk-borne and genetically transmitted mouse mammary tumor virus proviral DNA. Cell **19:**653-662.
5. **Desrosiers, R. C., C. Mulder, and B. Fleckenstein.** 1979. Methylation of herpesvirus saimiri DNA in lymphoid tumor cell lines. Proc. Natl. Acad. Sci. U.S.A. **76:**3839-3843.
6. **Doerfler, W.** 1969. Nonproductive infection of baby hamster kidney cells (BHK21) with adenovirus type 12. Virology **38:**587-606.
7. **Doerfler, W., U. Lundholm, and M. Hirsch-Kauffmann.** 1972. Intracellular forms of adenovirus DNA. I. Evidence for a DNA-protein complex in baby hamster kidney cells infected with adenovirus type 12. J. Virol. **9:**297-308.
8. **Doerfler, W., S. Stabel, H. Ibelgaufts, D. Sutter, R. Neumann, J. Groneberg, K. H. Scheidtmann, R. Deuring, and U. Winterhoff.** 1979. Selectivity in the integration sites of adenovirus DNA. Cold Spring Harbor Symp. Quant. Biol. **44:**551-564.
9. **Eick, D., S. Stabel, and W. Doerfler.** 1980. Revertants of adenovirus type 12-transformed hamster cell line T637 as tools in the analysis of integration patterns. J. Virol. **36:**41-49.
10. **Groneberg, J., and W. Doerfler.** 1979. Revertants of adenovirus type 12-transformed hamster cells have lost part of the viral genomes. Int. J. Cancer **24:**67-74.
11. **Groneberg, J., D. Sutter, H. Soboll, and W. Doerfler.** 1978. Morphological revertants of adenovirus type 12-transformed hamster cells. J. Gen. Virol. **40:**635-645.
12. **Guntaka, R. V., P. Y. Rao, S. A. Mitsialis, and R. Katz.** 1980. Modification of avian sarcoma proviral DNA sequences in nonpermissive XC cells but not in permissive chicken cells. J. Virol. **34:**569-572.
13. **Ibelgaufts, H., W. Doerfler, K. H. Scheidtmann, and W. Wechsler.** 1980. Adenovirus type 12-induced rat tumor cells of neuroepithelial origin: persistence and expression of the viral genome. J. Virol. **33:**423-437.
14. **Jones, K. W., J. Kinross, N. Maitland, and M. Norval.** 1979. Normal human tissues contain RNA and antigens related to infectious adenovirus type 2. Nature (London) **277:**274-279.
15. **Neumann, R., and W. Doerfler.** 1981. Integration of adenovirus type 2 DNA at a limited number of cellular sites in productively infected cells. J. Virol. **37:**887-892.
16. **Ortin, J., K.-H. Scheidtmann, R. Greenberg, M. Westphal, and W. Doerfler.** 1976. Transcription of the genome of adenovirus type 12. III. Maps of stable RNA from productively infected human cells and abortively infected and transformed hamster cells. J. Virol. **20:**355-372.
17. **Rigby, P. W. J., M. Diekmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113:**237-251.
18. **Sawada, Y., and K. Fujinaga.** 1980. Mapping of adenovirus 12 mRNAs transcribed from the transforming region. J. Virol. **36:**639-651.
19. **Scheidtmann, K. H., J. Ortin, and W. Doerfler.** 1975. Transcription of the genome of adenovirus type 12. II. Viral m-RNA in productively infected KB cells. Eur. J. Biochem. **58:**283-290.
20. **Sharp, P. A., P. H. Gallimore, and S. J. Flint.** 1974. Mapping of adenovirus 2 RNA sequences in lytically infected cells and transformed cell lines. Cold Spring Harbor Symp. Quant. Biol. **39:**457-474.
21. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503-517.
22. **Stabel, S., W. Doerfler, and R. R. Friis.** 1980. Integration sites of adenovirus type 12 DNA in transformed hamster cells and hamster tumor cells. J. Virol. **36:**22-40.
23. **Strohl, W. A., H. Rouse, K. Teets, and R. W. Schlesinger.** 1980. The response of BHK21 cells to infection with type 12 adenovirus. III. Transformation and restricted replication of superinfecting type 2 adenovirus. Arch. Gesamte Virusforsch. **31:**91-112.
24. **Sutter, D., and W. Doerfler.** 1979. Methylation of integrated viral DNA sequences in adenovirus type 12-transformed hamster cells. Cold Spring Harbor Symp. Quant. Biol. **44:**565-568.
25. **Sutter, D., and W. Doerfler.** 1980. Methylation of integrated adenovirus type 12 DNA sequences in transformed cells is inversely correlated with viral gene expression. Proc. Natl. Acad. Sci. U.S.A. **77:**253-256.
26. **Sutter, D., M. Westphal, and W. Doerfler.** 1978. Patterns of integration of viral DNA sequences in the genomes of adenovirus type 12-transformed hamster cells. Cell **14:**569-585.
27. **Tiemeier, D., L. Enquist, and P. Leder.** 1976. An improved derivative of a bacteriophage λ EK2 vector useful in the cloning of recombinant DNA molecules: λgtWES·λB. Nature (London) **263:**526-527.
28. **Tjia, S. T., E. B. Carstens, and W. Doerfler.** 1979. Infection of *Spodoptera frugiperda* cells with *Autographa californica* nuclear polyhedrosis virus. II. Kinetics of viral DNA replication. Virology **99:**399-409.
29. **Vardimon, L., D. Sutter, R. Neumann, I. Kuhlmann, and W. Doerfler.** 1980. Methylation of adenovirus DNA in transformed and infected cells. Nucleic Acids Res. **8:**2461-2473.
30. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76:** 3683-3687.
31. **Yoshida, K., and K. Fujinaga.** 1980. Unique species of mRNA from adenovirus type 7 early region 1 in cells transformed by adenovirus type 7 DNA fragment. J. Virol. **36:**337-352.
32. **zur Hausen, H.** 1973. Interactions of adenovirus type 12 with host cell chromosomes. Prog. Exp. Tumor Res. **18:** 240-259.