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Control of Simian Virus 40 Gene Expression in Adenovirus-Simian Virus 40 Hybrid Viruses

Synthesis of Hybrid Adenovirus 2-Simian Virus 40 RNA Molecules in Cells Infected with a Nondefective Adenovirus 2-Simian Virus 40 Hybrid Virus

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The effect of interferon on simian virus 40 (SV40) and adenovirus 2 (Ad2) T antigen synthesis has been examined in cells infected with SV40, with Ad2, and with a nondefective Ad2-SV40 hybrid virus, $Ad2^+ND_4$. The induction of SV40 T antigen by SV40 was highly sensitive to interferon, whereas the induction of Ad2 T-antigen by Ad2 was resistant. This difference in interferon sensitivity was also noted in cells simultaneously infected with both viruses. However, the induction of SV40 T antigen by $Ad2^+ND_4$, which contains covalently linked SV40 and Ad2 DNAs, was as resistant to interferon as the induction of Ad2 T antigen. This change in the interferon sensitivity of SV40 T antigen synthesis suggests that the expression of at least this portion of the SV40 genetic information in $Ad2^+ND_4$ is under Ad2 genetic control. When RNA extracted from $Ad2^+ND_4$-infected cells was examined by means of sequential hybridization with Ad2 DNA, elution, and rehybridization with SV40 DNA, 27% of the SV40-specific RNA was found to be linked to Ad2 RNA. No such linkage was detected in control mixtures of Ad2 and SV40 RNAs. The presence of Ad2 and SV40 nucleotide sequences in the same RNA molecule implies that, in $Ad2^+ND_4$ infection, transcription is initiated in the DNA of one virus (Ad2 or SV40) and continues without interruption across the point of junction into the DNA of the other virus. Furthermore, the interferon resistance of $Ad2^+ND_4$-induced SV40 T antigen synthesis suggests that transcription of the genetic information for SV40 T antigen is initiated in a region of Ad2 DNA.

Low doses of interferon markedly inhibit simian virus 40 (SV40) replication (37), SV40-specific antigen synthesis (33, 36, 37), and SV40-induced cell transformation (33, 49) without measurably affecting host cell synthetic processes (32, 34, 46; M. N. Oxman, M. J. Levin, D. H. Metz, K. Paucker, and M. A. Simpson, manuscript in preparation). This selective antiviral action has encouraged us to utilize interferon to examine the control of SV40 gene expression in SV40 transformed cells (32), in which the SV40 genome appears to be integrated into cellular DNA (45), and in other situations in which SV40 DNA may be linked to a heterologous DNA (31, 36).

Adenovirus (Ad)-SV40 hybrid viruses have been shown to contain SV40 DNA covalently linked to Ad DNA and to induce the synthesis of both SV40- and Ad-specific RNA and antigens in infected cells (1, 6, 7, 11, 13, 15, 16, 19, 21, 23-25, 30, 35, 40, 41, 43, 44). Since SV40 and Ad differ markedly in their sensitivities to interferon (8, 36), it was possible to determine whether this covalent linkage altered the interferon sensitivity of SV40 or Ad gene expression. Such studies, undertaken with several Ad-SV40 hybrid viruses, revealed that Ad T antigen induction by the hybrid viruses had the same low sensitivity to interferon as did Ad T antigen induction by wild-type Ad (36). In contrast, whereas SV40 T antigen induction by wild-type SV40 was very sensitive to interferon, even in cells simultaneously infected with SV40 and wild-type Ad, the interferon sensitivity of SV40 T antigen induction by the Ad-SV40 hybrid viruses was markedly decreased (36). In fact, the induction of this SV40-specific antigen by the Ad-SV40 hybrid viruses exhibited the same

low sensitivity to interferon as did the induction of Ad T antigens. These observations, which suggested that the expression of at least a portion of the SV40 genetic information in the hybrid virus genome was under Ad genetic control, led us to hypothesize that this functional linkage might be related to the synthesis of polycistronic RNA molecules containing both SV40 and Ad nucleotide sequences (31, 36).

We have now extended these interferon sensitivity studies to the nondefective Ad2-SV40 hybrid virus, $Ad2^{+}ND_4$ (24), and have examined the virus-specific RNA synthesized in $Ad2^{+}ND_4$-infected cells by means of sequential hybridization with Ad2 DNA, elution, and rehybridization with SV40 DNA. As was the case for the previously studied hybrid viruses, the induction of SV40 T antigen by $Ad2^{+}ND_4$ appears to be under Ad genetic control. Furthermore, a significant proportion of the SV40 RNA synthesized in $Ad2^{+}ND_4$-infected cells is covalently linked to Ad RNA. These findings suggest that transcription of at least some of the SV40 genetic information in this Ad2-SV40 hybrid virus is initiated in an adjoining region of Ad2 DNA, which may explain the observed alteration in the interferon sensitivity of hybrid virus-induced SV40 T antigen synthesis.

MATERIALS AND METHODS

Tissue culture. The Vero line (51) of African green monkey kidney (AGMK) cells was grown in Eagle minimal essential medium containing penicillin (250 U/ml), streptomycin (250 μg/ml), and 2 mM glutamine (EMEM) plus 10% fetal bovine serum (EMEM-10) and maintained in EMEM plus 2% fetal bovine serum (EMEM-2). The BSC-1 (12) and CV-1 (14, 42) lines of AGMK cells were grown in Dulbecco's modified Eagle medium containing the same concentrations of penicillin, streptomycin, and glutamine plus 10% fetal bovine serum and maintained in Dulbecco's modified Eagle medium plus 2% fetal bovine serum. Roller and stationary bottle cultures were refed biweekly until confluent monolayers were formed. Commercially obtained primary AGMK cells were grown in EMEM-10. All cell lines were repeatedly shown to be free of mycoplasma by anaerobic culture on Hayflick medium (10).

Viruses. SV40 (strain 777) (2) was produced in BSC-1 and CV-1 cells by low multiplicity (approximately 10^{-5} PFU/cell) infection, using an inoculum of virus from a single seed pool ($10^{9.3}$ PFU/ml) as previously described (35).

$Ad2^{+}ND_4$ (24) was grown in primary AGMK cells infected with a multiplicity of 1 to 5 PFU/cell. The virus pools used in these experiments represent the second passage after plaque purification. The derivation and biological properties of $Ad2^{+}ND_4$ have already been described (24).

Nonhybrid Ad2 (strain adenoid 6) was grown in suspension cultures of human (KB) cells and purified as previously described (11).

The Indiana strain of vesicular stomatitis virus was propagated in BSC-1 cells.

Interferon. Sendai virus-induced human leukocyte interferon (47, 48), partially purified by selective precipitation, was the generous gift of K. Cantell (State Serum Institute, Helsinki). By assay in human cells, this preparation contained 2×10^6 International Units/mg of protein (assayed in parallel with the British Standard of human interferon, code 69/19; Division of Biological Standards, National Institute for Medical Research, Mill Hill, London, U.K.). In Vero (monkey) cells, the antiviral activity of the human interferon proved to be approximately 1/25 of that in human cells. Since all of our experiments were performed in Vero cells, a unit of interferon is defined here as the amount required to produce a 50% reduction in vesicular stomatitis virus plaque formation in the Vero cells employed. This corresponds to approximately 25 international units.

To determine the effect of interferon on SV40 and Ad T antigen formation, confluent monolayers of Vero cells in 35-mm plastic petri dishes (Falcon Plastics) containing glass cover slips were exposed for 18 to 20 h at 37 C to 2 ml of various concentrations of interferon diluted in EMEM-2. The monolayers, each of which contained approximately 10^6 cells, were then thrice washed with 2 ml of EMEM-2 and infected with 1 ml of EMEM-2 containing either SV40 alone (4.5×10^8 PFU), nonhybrid Ad2 alone (3.7×10^7 PFU), SV40 (4.5×10^8 PFU) plus nonhybrid Ad2 (3.7×10^7 PFU), or $Ad2^{+}ND_4$ (1.6×10^7 PFU). After 2 h, an additional 1 ml of EMEM-2 was added and incubation continued at 37 C. Cover slips were fixed 25 h after infection and stored at −70 C for subsequent fluorescent antibody (FA) staining. Two or three replicate cultures were used for each point.

T antigen assays. SV40 and Ad2 T antigens were assayed by an indirect FA procedure using serum pools from hamsters bearing virus-free transplants of virus-induced tumors and fluorescein-conjugated goat antihamster globulin (Antibodies Inc., Davis, Calif.). The Ad2 T antiserum was obtained from hamsters bearing tumors induced by hamster kidney cells transformed by nonhybrid Ad2 (strain $Ad2^{-}$) (15; A. M. Lewis, Jr., A. S. Levine, and A. S. Rabson, manuscript in preparation). Cover slips were bisected so that one-half could be stained for SV40 T antigen and the other half stained for Ad2 T antigen. Procedures for fixing and staining have been described elsewhere (39). Quantitation was accomplished by inserting a disk with a small central hole into the microscope ocular to reduce the size of the field so that under high power it contained only 10 to 20 cells. Both positive and negative cells (a total of at least 1,000) were counted in 50 to 100 such fields evenly distributed over each cover slip.

Preparation of virus and extraction of viral DNA. Both Ad2 and SV40 were purified from cells and medium by a previously described (35) modification of the technique of Burnett et al. (4). After cushioning onto CsCl (density = 1.45 g/ml), the viruses were further purified by two cycles of equilibrium density gradient centrifugation in CsCl (density = 1.34 g/ml).

DNA was extracted from purified virions by digestion with 1 mg of self-digested Pronase per ml (B grade, nuclease free; Calbiochem) in the presence of 3 mg of sodium dodecyl sulfate (SDS) per ml, followed by phenol extraction and dialysis against 0.1 × SSC (SSC = 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 6.9) (16). SV40 DNA was further purified by two cycles of equilibrium density centrifugation in CsCl (density = 1.60 g/ml) containing 200 μg of ethidium bromide per ml. The form I SV40 DNA, which constituted 90 to 95% of the DNA extracted from the purified SV40 virions, was extracted twice with an equal volume of a CsCl-saturated solution consisting of 9 parts isopropyl alcohol and 1 part 0.1 × SSC. The DNA was then dialyzed against 0.01 × SSC at 4 C and stored at −70 C. This SV40 DNA was free of detectable host cell (monkey) nucleotide sequences as determined by sensitive hybridization assays (35).

Escherichia coli DNA was extracted by the method of Marmur (26). The concentrations of DNA solutions were determined by a modified diphenylamine reaction (5) with calf thymus DNA (Calbiochem) as a standard.

Preparation of radiolabeled RNA. Radiolabeled RNA from $Ad2^{+}ND_4$- and Ad2-infected Vero cells was prepared from confluent bottle cultures (32 oz, about 900 ml) infected with 20 to 50 PFU of these viruses per cell in 10 ml of EMEM-2. Four hours postinfection, 15 ml of EMEM-2 containing sufficient uridine-5-^{3}H (New England Nuclear Corp.; 25 to 30 Ci/mmol) to yield a final concentration of 100 μCi/ml was added. The cells were harvested by scraping 24 h after infection, and washed cell pellets were stored at −70 C for subsequent RNA extraction.

Radiolabeled early SV40 RNA (SV40-specific RNA transcribed from the DNA of input virions in the absence of SV40 DNA replication) and late SV40 RNA (SV40-specific RNA transcribed after SV40 DNA replication has commenced) were prepared in roller bottle cultures of Vero cells as previously described (35). For the preparation of early SV40 RNA, 20 μg of cytosine arabinoside per ml was present throughout infection to prevent SV40 DNA replication (35).

RNA was extracted by a hot phenol-SDS procedure (20) and stored at −70 C in 2 × SSC plus 0.05% SDS. RNA concentrations were determined with the orcinol reaction (3), with yeast-soluble RNA (Calbiochem) as a standard.

RNA-DNA hybridization. Radiolabeled RNA was hybridized with Ad2 or SV40 DNA immobilized on nitrocellulose membrane filters (Millipore Corp.; 0.45 μm HAWP) (9, 20). The hybridization reaction was either carried out in a solution consisting of equal parts of formamide and 10 × SSC for 18 h at 37 C, or in 2 × SSC + 0.05% SDS for 18 h at 60 C. Filters were then removed and extensively washed with 2 × SSC, first at 37 C and then at 60 C. Filters not destined for elution were then treated with pancreatic ribonuclease (20 μg/ml) and ribonuclease T1 (10 U/ml) for 1 h at room temperature, washed again with 2 × SSC at 60 C, and counted in a Packard Tri-Carb liquid scintillation spectrometer. "Blank" filters containing an equivalent amount of *E. coli* DNA were included in each hybridization reaction.

Elution and rehybridization of virus-specific RNA. After washing with 2 × SSC at 60 C, filters from the hybridization reaction were eluted with 250 μliters of elution mixture (formamide-0.01 × SSC, 9:1) for 30 min at 37 C. The eluent was removed, and 250 μliters of fresh elution mixture was added for 45 min at 37 C; the temperature was then raised to 45 C for an additional 15 min. This procedure eluted 65 to 80% of the [^{3}H]RNA initially bound to the filters. To recover the remaining [^{3}H]RNA, the filters were eluted with 1 ml of 0.01 × SSC at 100 C for 15 min. The two formamide eluents were pooled, adjusted with concentrated SSC so that the formamide-10 × SSC ratio was 1:1, and rehybridized with fresh DNA filters for 18 h at 37 C. The [^{3}H]RNA eluted by boiling was precipitated at −30 C after the addition of 200 μg of poly I:C, 0.1 ml of 3 M NaCl, and 2 volumes of ethanol. The [^{3}H]RNA was then collected by centrifugation, resuspended in 2 × SSC + 0.05% SDS, and rehybridized with fresh DNA filters for 18 h at 60 C. All of the rehybridized filters were RNase treated before counting, and the SV40-specific counts per minute was determined by subtracting the counts per minute bound to the *E. coli* DNA blanks from the counts per minute bound to the SV40 DNA filters. The counts per minute of SV40-specific RNA recovered after elution and rehybridization with SV40 DNA (Tables 1 and 2) represents the sum of the SV40-

TABLE 1. *Detection of hybrid RNA molecules containing both SV40 and AD2 nucleotide sequences*

RNA	Virus-specific [^{3}H]RNA counts/min initially present[a]		Recovery of SV40-specific RNA after hybridization with Ad2 DNA, elution, and rehybridization with SV40 DNA	
	SV40	Ad2	Counts/min	Recovery (%)[b]
Early SV40	320	0	0	0
Early SV40	750	0	0	0
Early SV40	11,724	0	27	0.23
Late SV40	5,100	0	0	0
Ad2	0	170,000	26	<0.02
$Ad2^{+}ND_4$	446	NT[c]	42	9.4
$Ad2^{+}ND_4$	900	4,500	54	6.0
$Ad2^{+}ND_4$	24,940	80,360	1,720	6.9

[a] Each number represents the average of duplicate or triplicate determinations. The virus-specific counts per minute is the net of the counts per minute bound to 1 μg SV40 or Ad2 DNA filters minus the counts per minute bound to 1 μg *E. coli* DNA filters. In all cases, the counts per minute bound to the *E. coli* blank was less than one-eighth that bound to the viral DNA filters.

[b] Expressed as (recovered SV40-specific counts per minute/initially present SV40-specific counts per minute) × 100. The net SV40-specific counts per minute was determined with 1μg SV40 and *E. coli* DNA filters.

[c] NT, not tested.

TABLE 2. *Detection of hybrid RNA molecules containing both SV40 and Ad2 nucleotide sequences*

RNA	Virus-specific [^{3}H]RNA counts/min initially present[a]		First filter (2 μg of DNA)	Removal of SV40-specific RNA (%)[b]	Recovery of SV40-specific RNA after hybridization with first filter, elution, and rehybridization with SV40 DNA	
	SV40	Ad2			Counts/min	Recovery (%)[c]
Early SV40 + Ad2[d]	8,440	429,000	Ad2	0	1	<0.02
			SV40	88	1,834	21.8
			E. coli	0	2	<0.03
$Ad2^+ND_4$	15,450	114,000	Ad2	53	1,736	11.2
			SV40	68	6,364	41.2
			E. coli	0	6	<0.04

[a] Each number represents the average of duplicate or triplicate determinations. The virus-specific counts per minute is the net of the counts per minute bound to 2 μg of SV40 or Ad2 DNA filters minus the counts per minute bound to 2 μg of *E. coli* DNA (blank) filters. In all cases, the counts per minute bound to the *E. coli* blank was less than one-tenth that bound to the viral DNA filters.

[b] Expressed as [(initial SV40-specific counts per minute − remaining SV40-specific CPM)/(initial SV40-specific counts per minute)] × 100. The remaining SV40-specific counts per minute was determined by adding a fresh 2-μg SV40 DNA filter to the hybridization fluid remaining from the initial hybridization.

[c] Expressed as (recovered SV40-specific counts per minute/initially present SV40-specific counts per minute) × 100. The net SV40-specific counts per minute was determined with 1 μg SV40 and *E. coli* DNA filters.

[d] Early SV40 RNA and Ad2 RNA were separately extracted and mixed in 2 × SSC + 0.05% SDS.

specific counts per minute recovered from the formamide eluents and from the 0.01 × SSC eluents.

RESULTS

Interferon sensitivity of SV40 and Ad2 T antigen formation in Vero cells infected with SV40, with Ad2, and with mixtures of SV40 and Ad2. In cultures infected with SV40 alone, in the absence of interferon, more than 99% of the cells contained SV40 T antigen. However, in replicate cultures, even the lowest dose of interferon used (1 U/ml) produced a significant reduction in the proportion of T antigen positive cells, and a dose of 7 U/ml resulted in 95% inhibition of SV40 T antigen formation (Fig. 1). This reduction in the proportion of T antigen positive cells was roughly paralleled by a reduction in the intensity of FA staining in those few cells which were positive.

Simultaneous infection with nonhybrid Ad2 did not change the proportion of SV40 T antigen positive cells, nor did it affect the intensity or morphology of SV40 T antigen staining. Of more importance, the interferon sensitivity of SV40-induced SV40 T antigen formation was not altered in cells simultaneously infected with Ad2.

The synthesis of Ad2 T antigen in Ad2-infected cells was relatively resistant to interferon, and this was not altered by simultaneous infection with SV40 (Fig. 1). Pretreatment with 50 U of interferon per ml had no measureable effect, and 200 U/ml produced less than a 50% inhibition of Ad2 T antigen formation.

Interferon sensitivity of SV40 and Ad2 T antigen formation in Vero cells infected with $Ad2^+ND_4$. In cultures infected with $Ad2^+ND_4$, in the absence of interferon, approximately 90% of the cells contained SV40 T antigen. However, in contrast to wild-type SV40 infection, doses of interferon as high as 50 U/ml did not reduce the proportion of SV40 T antigen positive cells or alter the intensity or morphology of FA staining (Fig. 1). Pretreatment with 200 U/ml did produce a moderate inhibition of SV40 T antigen formation. Thus, more than 100 times as much interferon was required to inhibit SV40 T antigen induction by $Ad2^+ND_4$ than was required to inhibit SV40 T antigen induction by SV40, indicating that the induction of SV40 T antigen by this hybrid virus is more than 100-fold less sensitive to interferon than is the induction of the same SV40 antigen by wild-type SV40.

In contrast to the marked difference in the interferon sensitivity of SV40 T antigen induction by wild-type SV40 and by $Ad2^+ND_4$, there was little difference in the interferon sensitivity of Ad2 T antigen induction by wild-type Ad2 and by $Ad2^+ND_4$. Ad2 T antigen formation by

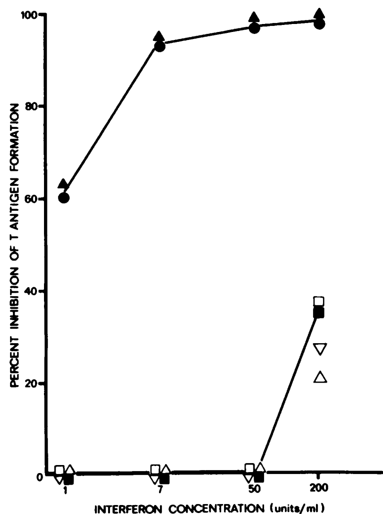

FIG. 1. *Interferon sensitivity of Ad2 and SV40 T antigen formation in cells infected with SV40 alone, Ad2 alone, a mixture of SV40 and Ad2, and Ad2⁺ND₄. Symbols: ●, SV40 infection, SV40 T antigen measured: ▽, Ad2 infection, Ad2 T antigen measured; ▲, SV40 and Ad2 infection, SV40 T antigen measured; △, SV40 and Ad2 infection, Ad2 T antigen measured; ■, Ad2⁺ND₄ infection, SV40 T antigen measured; □, Ad2⁺ND₄ infection, Ad2 T antigen measured. Confluent cover-slip cultures of Vero cells were pretreated with interferon, infected, harvested, and FA-stained for SV40 and Ad2 T antigens as described in Materials and Methods. Percent inhibition of T antigen formation = [(percent of cells positive in control culture − percent of cells positive in treated culture)/(percent of cells positive in control culture)] × 100.*

$Ad2^+ND_4$ was relatively resistant to interferon (Fig. 1). In fact, the $Ad2^+ND_4$-induced synthesis of both the SV40 and the Ad2 T antigens showed comparable sensitivity to interferon (Fig. 1).

Detection of RNA molecules containing both SV40 and Ad2 nucleotide sequences. The observations detailed above suggest that the expression of at least that portion of the SV40 genetic information in $Ad2^+ND_4$ which is responsible for SV40 T antigen synthesis is under Ad genetic control. Since in SV40 infection interferon appears to act at the level of transcription (27, 34; D. H. Metz, M. N. Oxman, and M. J. Levin, manuscript in preparation), a likely mechanism for such control would be the initiation of the transcription of these SV40 sequences in an adjacent region of Ad2 DNA, with the synthesis of polycistronic RNA molecules containing covalently linked Ad2 and SV40 nucleotide sequences. Such hybrid RNA molecules should be able to hybridize sequentially to both Ad2 and SV40 DNA, and consequently we sought to detect them by hybridizing [^{3}H]RNA from $Ad2^+ND_4$-infected cells to Ad2 DNA and then eluting the hybridized RNA and rehybridizing it to SV40 DNA.

Table 1 summarizes the results obtained in three experiments. In each of them a significant proportion (6 to 9.4%) of the SV40-specific counts per minute initially present in $Ad2^+ND_4$ RNA hybridized to Ad2 DNA and could then be eluted and rehybridized to SV40 DNA. Control preparations of [^{3}H]RNA from Ad2-infected cells, or from cells infected with SV40 in the presence (early SV40 RNA) or absence (late SV40 RNA) of cytosine arabinoside, did not hybridize sequentially with both Ad2 and SV40 DNA. Early SV40 RNA was used as a control because the SV40-specific RNA induced by $Ad2^+ND_4$ consists primarily of early SV40 RNA sequences (17, 21).

In a final experiment, a mixture of early SV40 RNA and Ad2 RNA was compared with the RNA extracted from $Ad2^+ND_4$-infected cells (Table 2). When the mixture was hybridized with Ad2 or *E. coli* DNA and the filters were eluted, the eluents contained no SV40-specific RNA. As expected, an SV40 filter retained a significant amount of SV40 RNA, which could then be eluted and detected on rehybridization with SV40 DNA; 21.8% of the SV40-specific counts per minute initially present in the mixture was recovered as RNase-resistant counts per minute on the final SV40 DNA filter.

When $Ad2^+ND_4$ RNA was similarly examined, both Ad2 DNA and SV40 DNA filters retained SV40-specific RNA, which could then be eluted and rehybridized with SV40 DNA. When the first filter contained SV40 DNA, 41.2% of the SV40-specific counts per minute initially present was recovered as RNase-resistant counts per minute on the final SV40 DNA filter. The discrepancy between this figure and the 100% recovery expected on a theoretical basis may be explained by incomplete removal of SV40-specific counts per minute by the first filter (Table 2), together with losses occurring during the elution and rehybridization procedures. When the first filter contained Ad2 DNA, 11.2% of the SV40-specific counts per minute initially present was recovered as RNase-resistant counts per minute on the final SV40 DNA filter. A comparison of this recovery with that obtained when both the first and final filters contained SV40 DNA indicates that at least 27% ([11.2/41.2] × 100) of the SV40-specific [^{3}H]RNA counts per minute synthesized in $Ad2^+ND_4$-infected cells is covalently linked to Ad2 RNA.

This conclusion was further supported by an examination of the fluids remaining after the

initial hybridization (Table 2). With the mixture of early SV40 RNA and Ad2 RNA, only hybridization with SV40 DNA removed a significant number of SV40-specific counts per minute, whereas in the case of $Ad2^+ND_4$ RNA, SV40-specific counts per minute were removed by hybridization with both Ad2 and SV40 DNA.

DISCUSSION

Previous studies using RNA-DNA hybridization and heteroduplex mapping have demonstrated that $Ad2^+ND_4$ contains 43% of the wild-type SV40 genome covalently linked to Ad2 DNA (11, 15). This SV40 genetic information, which includes all of the early SV40 nucleotide sequences (22), is inserted as a single segment of SV40 DNA at a point approximately 14% in from one end of the Ad2 genome (15). The fact that a significant proportion of the SV40-specific RNA synthesized during $Ad2^+ND_4$ infection could be hybridized to Ad2 DNA, eluted, and rehybridized to SV40 DNA (Tables 1 and 2) indicates that at least some of the SV40 RNA sequences induced by this hybrid virus are contained in polycistronic RNA molecules which also contain Ad2 nucleotide sequences. Unless we suppose that separate Ad2 and SV40 RNA molecules are covalently joined at some time after transcription, the presence of Ad2 and SV40 nucleotide sequences in the same RNA molecule implies that transcription is initiated in the DNA of one virus (Ad2 or SV40) and continues without interruption across the point of juncture into the DNA of the other virus. Furthermore, the results of the interferon studies reported here (Fig. 1) provide evidence that transcription of the genetic information for SV40 T antigen is initiated in a region of Ad2 DNA. It requires more than 100 times as much interferon to block the synthesis of SV40 T antigen when it is induced by $Ad2^+ND_4$ than when it is induced by wild-type SV40. In fact, the interferon resistances of SV40 and Ad2 T antigen induction by this hybrid virus are nearly identical (Fig. 1). Since the interferon sensitivity of SV40 T antigen induction by wild-type SV40 is not altered in cells simultaneously infected with nonhybrid Ad2, the decreased interferon sensitivity of $Ad2^+ND_4$-induced SV40 T antigen synthesis cannot be explained by a diffusible Ad2-induced substance or by some Ad2-induced alteration in cell metabolism. Rather, it would appear to be a consequence of the covalent linkage of SV40 and Ad2 DNA in the $Ad2^+ND_4$ genome and to reflect the control of SV40 gene expression by adjacent Ad2 genetic information. Although the mechanism of action of interferon continues to be the subject of debate (46), recent observations suggest that, in SV40 infection, interferon acts by blocking transcription (27, 34; D. H. Metz, M. N. Oxman, and M. J. Levin, manuscript in preparation). If this is also true of Ad-SV40 hybrid viruses, the alteration in the interferon sensitivity of $Ad2^+ND_4$-induced SV40 T antigen synthesis indicates that transcription of at least this portion of the SV40 genetic information in $Ad2^+ND_4$ is controlled by the Ad2 genome. Furthermore, in the event that the site of interferon action is the initiation of transcription (D. H. Metz, M. N. Oxman, and M. J. Levin, manuscript in preparation), our observations imply that transcription of the SV40 portion of the $Ad2^+ND_4$ genome is initiated in an adjacent region of Ad2 DNA. This would explain both the synthesis of hybrid RNA molecules in $Ad2^+ND_4$-infected cells and the relative resistance to interferon of $Ad2^+ND_4$-induced SV40 T antigen synthesis.

This model is further supported by observations on the effect of interferon on SV40-specific RNA synthesis in Vero cells infected with SV40 and with a nondefective Ad2-SV40 hybrid virus ($Ad2^+ND_1$) which is closely related to $Ad2^+ND_4$ (24). Interferon (50 to 100 U/ml) reduces the level of early SV40 RNA in SV40-infected Vero cells by more than 90% (34). When interferon-treated cells are pulse-labeled and fractionated, a comparable reduction in early SV40 RNA is observed in the nucleus and in the cytoplasm, a result consistent with a decrease in transcription rather than an increase in the cytoplasmic breakdown of untranslated RNA (D. H. Metz, M. N. Oxman, and M. J. Levin, manuscript in preparation). In contrast, a similar dose of interferon does not measurably affect the synthesis of SV40-specific RNA in $Ad2^+ND_1$-infected cells (M. J. Levin, M. N. Oxman, and A. M. Lewis, Jr., unpublished observations). Thus the transcription of the SV40 genetic information in this hybrid virus appears to be resistant to interferon, whereas the transcription of the wild-type SV40 genome is sensitive. Furthermore, by using hybridization, elution, and rehybridization techniques similar to those described here, Fox and Baum have detected the synthesis of RNA molecules containing both Ad and SV40 nucleotide sequences in cells infected with an Ad7-SV40 hybrid virus, $E46^+$ (R. I. Fox and S. G. Baum, personal communication). Like $Ad2^+ND_4$, $E46^+$ induces the synthesis of SV40 T antigen (13, 40, 43), and $E46^+$-induced SV40 T antigen synthesis is likewise highly resistant to interferon (36).

A comparable alteration in the interferon sensitivity of SV40 T antigen synthesis has also been observed in SV40-transformed 3T3 cells (32), in which the SV40 genome is thought to be

covalently linked to cellular DNA (45). The synthesis of SV40 T antigen by these cells is refractory to interferon (32), and interferon concentrations in excess of 500 U/ml do not measurably affect their synthesis of SV40-specific RNA (M. N. Oxman, M. J. Levin, D. H. Metz, K. Paucker, and M. A. Simpson, manuscript in preparation). This contrasts with the marked inhibitory effect of interferon on both SV40 T antigen formation and SV40-specific RNA synthesis observed during lytic infection (34, 36). Since the expression of cellular genes in these cells is unaffected by interferon (M. N. Oxman, M. J. Levin, D. H. Metz, K. Paucker, and M. A. Simpson, manuscript in preparation), the failure of interferon to inhibit their synthesis of SV40 T antigen suggests that the expression of this SV40 genetic information is under the control of the cell genome. The reported presence of both cell- and virus-specific sequences in the same molecules of nuclear RNA from SV40-transformed 3T3 cells (50) is consistent with the hypothesis that the transcription of the integrated SV40 DNA is initiated in a region of adjacent cell DNA (31, 32).

The results of heteroduplex mapping (15, 28, 29) and hybridization competition (21) studies of $Ad2^+ND_4$ and the four other closely related, nondefective Ad2-SV40 hybrid viruses (24) have revealed the relative positions of the three identifiable early SV40 genetic functions in the $Ad2^+ND_4$ genome. In addition, Morrow et al. (28) have shown that the SV40 DNA segment in $Ad2^+ND_4$ is colinear with wild-type SV40 DNA, and Khoury et al. (18) have presented data which indicate that, during SV40 infection, the direction of transcription of the early region of the SV40 genome is from the T antigen locus toward the U antigen locus. Patch et al. (38; C. T. Patch, A. M. Lewis, Jr. and A. S. Levine, submitted for publication) have compared the hybridization of complementary SV40 RNA and early SV40 RNA to the separated DNA strands of all five nondefective Ad2-SV40 hybrid viruses and have concluded that the template for early SV40 RNA is in the light strand of each of them. Since the SV40-specific RNA in $Ad2^+ND_4$-infected cells hybridizes extensively with the (−) strand of SV40 DNA (the early template) and not with the (+) strand (17), it must be transcribed from the light strand of $Ad2^+ND_4$ DNA. Our demonstration of the synthesis in $Ad2^+ND_4$-infected cells of Ad2-SV40 hybrid RNA molecules would appear to provide direct evidence that transcription is continuous across a point of juncture of Ad2 and SV40 DNA, either being initiated in SV40 DNA and extending into Ad2 DNA or being initiated in Ad2 DNA and extending into SV40 DNA. The alteration in the interferon sensitivity of $Ad2^+ND_4$-induced SV40 T antigen synthesis (as well as the resistance of $Ad2^+ND_1$-induced SV40-specific RNA synthesis to interferon) argues strongly in favor of initiation in Ad2 DNA. Initiation in Ad2 DNA is supported by other observations as well. The entire region of the SV40 (—) strand in each of the five nondefective Ad2-SV40 hybrid viruses appears to be transcribed during lytic infection (17), whereas only $Ad2^+ND_4$ contains the region associated with SV40 T antigen induction (21, 24). Thus, unless the U and TSTA regions of the other hybrid viruses have their own promoters, it seems likely that transcription of the SV40 (—) strand segment in each of the hybrid viruses is initiated in Ad2 DNA upstream from the SV40 early antigen region (17). Taken together, all of these observations can be represented by the following model (Fig. 2). Transcription of the SV40 T antigen locus is initiated at a site in the larger Ad2 DNA segment of the light strand of $Ad2^+ND_4$ DNA. This results in the synthesis of a polycistronic RNA molecule containing Ad2 sequences at the 5′ end. It is not yet clear, however, whether this transcript includes all of the SV40 genetic information in the hybrid virus genome. The examination of the SV40 and Ad2 sequences in the hybrid RNA molecules synthesized in $Ad2^+ND_4$-infected cells, particularly those at the 5′ end, should enable us to test the hypothesis that the transcription of SV40 genetic information in $Ad2^+ND_4$ is initiated in a region of Ad2 DNA and to determine the location of that initiation site in the $Ad2^+ND_4$ genome. Moreover, the identification of the SV40 sequences in these hybrid RNA molecules should reveal whether all of the early SV40 genetic information is transcribed as a single unit. Finally, a comparison of the 5′ terminal sequences of these hybrid RNA molecules with those of early SV40 RNA may reveal the basis for the difference in the sensitivities of Ad and SV40 to interferon.

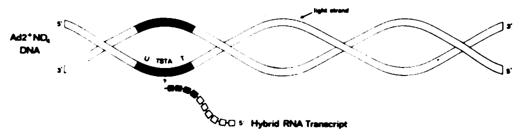

FIG. 2. *Model for the transcription of polycistronic RNA molecules containing covalently linked Ad2 and SV40 RNA from the DNA of $Ad2^+ND_4$. The observations upon which this model is based are detailed in the Discussion.*

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