Proc. Nat. Acad. Sci. USA
Vol. 72, No. 2, pp. 753–757, February 1975

Hamster α-Amanitine-Resistant RNA Polymerase II Able to Transcribe Polyoma Virus Genome in Somatic Cell Hybrids

(interspecific hybrids/dominance/susceptibility to polyoma)

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Communicated by Renato Dulbecco, November 15, 1974

ABSTRACT **A hamster cell line resistant to α-amanitine has been isolated (α-am^r, BHK-T6-G$_1$). Cell extracts of this mutant have an α-amanitine-resistant RNA polymerase II (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) activity as shown by DEAE-cellulose column chromatography. This mutation is dominant in interspecific hybrids with 3T3 mouse cells. In such hybrids polyoma virus can grow with equal efficiency in the presence or absence of the drug, thus indicating that the RNA polymerase of the unsusceptible parental cell can participate in the correct transcription of the viral genome.**

Polyoma virus can grow on 3T3 mouse cells and transform BHK hamster cells. It can also grow on somatic cell hybrids between these two lines (1), thus demonstrating that susceptibility to virus growth is dominant over lack of susceptibility. In transformed cells, the genome of the virus is only partially transcribed (2, 3), suggesting that the difference between susceptible and unsusceptible cells may reside in the differential transcription ability of the two cell lines. This hypothesis can be tested in polyoma-infected somatic cell hybrids of susceptible and unsusceptible cells, under conditions in which the RNA polymerase (nucleosidetriphosphate: RNA nucleotidyltransferase, EC 2.7.7.6) of the susceptible cell is inhibited. It is known, in fact, that α-amanitine is a specific inhibitor of RNA polymerase II (4) and that it prevents simian virus 40 (SV40) growth by inhibiting the transcription in permissive cells (5). On the other hand, α-amanitine-resistant mutants can be isolated in hamster cells (6). Thus, if polyoma, like SV40, is dependent on RNA polymerase II for its transcription in permissive cells, the conditions for testing the above hypothesis are available.

We have isolated and characterized an α-amanitine-resistant mutant of BHK-T6 hamster cell line altered in RNA polymerase II. The resistant phenotype is dominant in hybrids with 3T3-TK$^-$ cells. In the presence of the drug, polyoma cannot grow on 3T3-TK$^-$ cells, whereas it grows normally on this hybrid. Therefore, the present work indicates that the limitation of the growth of this virus in unsusceptible cells is likely to be at a level other than the host RNA polymerase.

MATERIALS AND METHODS

BHK-T6 cells are mutants deficient in hypoxanthine guanine phosphoribosyltransferase (HGPRT), isolated by Marin and Littlefield (7). Mouse 3T3-TK$^-$, a thymidine-kinase-deficient line, was obtained from Basilico *et al.* (8).

Media and growth conditions for independent cells lines as well as for selection of hybrids were already described (9).

Polyoma virus was propagated on 3T3 cells by standard methods. Multiplicity of infection (MOI) is in terms of plaque-forming units (PFU).

In vitro RNA polymerase activity was assayed as previously described (6).

Fresh α-amanitine (C. H. Boehringer, Ingelheim) was prepared weekly by dissolving the powder in sterile water and keeping the concentrated solution at −60° until use.

Amphotericin B (Fungizone, GIBCO) solution was prepared by dissolving the lyophilized powder in sterile water and was added to the medium at sub-inhibitory concentration depending on the cell line in use (10).

Rifampicin S.A. (state of the art) was purchased from Sigma Chemical Co.

RESULTS

(*a*) *Isolation of α-Amanitine-Resistant BHK-T6 Cells.* Semi-confluent cultures of hamster BHK-T6 cells were trypsinized and replated in medium containing 1 μg/ml of α-amanitine and 10 μg/ml of amphotericin B. It has been demonstrated that the addition of sublethal doses of amphotericin B improves cell permeability to unrelated drugs (11); this synergistic action seems to be due to the formation of holes on the cell membrane (12). The medium was changed every 48 hr; after the third change a few micro-colonies were already present in each plate (approximately 0.8 to 1.2 × 10^{-6} per input cell). Subsequently medium was changed every 4 days until colonies of 0.3- to 0.4-cm diameter were formed. From independent plates a few colonies were picked and replated until mass cell cultures were obtained. Approximately 30 independent clones of α-amanitine-resistant cells were thus obtained. Five of these cultures were grown again from 10^3 to 10^7 cells in the absence of α-amanitine and amphotericin B and then tested for growth in the presence of 5 μg/ml of α-amanitine with or without amphotericin B. All of them proved to be resistant to this concentration of α-amanitine. One of these lines (BHK-T6-G$_1$) was further cloned, retested, and used in the subsequent experiments.

We shall call the mutation selected α-am^r as opposed to the α-am^s wild-type allele.

(*b*) *Evidence for a Modified α-am^r RNA Polymerase of BHK-T6-G$_1$ Cells.* To verify that the isolated mutant was really the

Abbreviations: SV40, simian virus 40; MOI, multiplicity of infection.

TABLE 1. *Effect of α-amanitine on the ammonium-sulfate-stimulated RNA polymerase activity in nuclei of BHK-T6 and BHK-T6-G$_1$ cells**

Nuclei from†	Ammonium sulfate (0.27 M)	α-Amanitine (3.3 μg/ml)	[^{3}H]UMP incorporated‡ cpm	%
BHK-T6	−	−	4260	100
BHK-T6	−	+	2910	68
BHK-T6	+	−	8500	100
BHK-T6	+	+	1100	12
BHK-T6-G$_1$	−	−	1490	100
BHK-T6-G$_1$	−	+	1410	94
BHK-T6-G$_1$	+	−	2580	100
BHK-T6-G$_1$	+	+	2680	103

* RNA polymerase activity is measured by following the incorporation of [^{3}H]UMP into trichloroacetic-acid-precipitable material. Standard assay mixtures (0.10 ml) contain, as described by Jackson and Sugden (5): 4% (w/v) glycerol, 1 mM dithiothreitol, 10 mM $MgCl_2$, 10 mM Tris·HCl, pH 7.9, 50 mM ATP, 1 mM CTP and GTP, 30 μCi of [^{3}H]UTP (46 Ci/mmol; Radiochemical Centre, Amersham, England), and, where indicated, ammonium sulfate and α-amanitine. Reactions are started by the addition of 0.05 ml of nuclear suspension (about 1 to 5 × 10^6 nuclei) to the prewarmed assay mixtures. Reactions are incubated at 37° for 30 min, then stopped by the addition of 2 ml of 12% trichloroacetic acid containing 10 mM potassium pyrophosphate. Precipitates are collected on nitrocellulose filters (Millipore, HAWP, 0.45 μm, 25 mm) and filters are washed with 50 ml of 5% trichloroacetic acid containing 10 mM potassium pyrophosphate and their radioactivity is measured in Bray's scintillation fluid (14). A zero-time blank was carried out in each experiment. Experiments were carried out in duplicate.

† Nuclei are prepared from the indicated cell lines, according to the procedure of Jackson and Sugden (5).

‡ Radioactivity incorporated in 30 min of incubation, minus the zero-time blank. Values represent the average of duplicate determinations. Gross differences between incorporation of BHK-T6 and BHK-T6-G1 nuclei are due to different concentrations of nuclei in the nuclear suspensions.

carrier of an altered RNA polymerase II, we studied the effect of α-amanitine on the endogenous incorporation of [^{3}H]UMP in isolated nuclei prepared by the method described by Jackson and Sugden (5) from BHK-T6 and BHK-T6-G$_1$ cells.

TABLE 2. *Properties of the nucleoplasmic RNA polymerase (pool II of DEAE-Sephadex)*

	Source	
	BHK-T6	BHK-T6-G$_1$
% inhibition by 1 μg/ml of α-amanitine*	70	0
% inhibition by 6 μg/ml of rifampicin*	32	24
Optimum ammonium sulfate concentration†	100 mM	100 mM
Optimum $MnCl_2$ concentration‡	0.5–1.5 mM	0.5–1.0 mM

* The RNA polymerase assay was carried out at 100 mM ammonium sulfate and 1 mM $MnCl_2$.

† Carried out at 1 mM $MnCl_2$.

‡ Carried out at 100 mM ammonium sulfate.

It is known that α-amanitine is a potent inhibitor of one of the nuclear RNA polymerases, form II (or form B), while it has no effect on form I or III (13). Table 1 reports the results of this test, showing that nuclei from BHK-T6-G1 are capable of RNA synthesis in the presence of α-amanitine, while in the BHK-T6 nuclei this synthesis is strongly inhibited by the drug. α-Amanitine-sensitive RNA synthesis in isolated nuclei of the BHK-T6-G$_1$ hybrid is activated by ammonium ions (5); since in the nuclei the ammonium sulfate-stimulated [^{3}H]UMP incorporation is not inhibited by α-amanitine, we infer that this mutation depends on an altered RNA polymerase II.

(c) Analysis of the RNA Polymerase II of BHK-T6 and BHK-T6-G$_1$ Cells. DNA-dependent RNA polymerases were solubilized from whole cells in hypotonic buffer according to Sugden and Keller (15). Solubilized proteins were concentrated by ammonium sulfate precipitation, dissolved in buffer DEAE (50 mM Tris·HCl, pH 7.9, containing 0.1 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol, and 40 mM ammonium sulfate), and dialyzed against several changes of the same buffer. Insoluble material was removed by high-speed centrifugation and the sample was applied to a column (1 × 7-cm) of DEAE-Sephadex A-25 equilibrated with the same buffer. The column was washed with two volumes of buffer DEAE and a linear gradient of 40–400 mM ammonium sulfate (20 ml in each solution) in buffer was applied. Fractions (0.80 ml), were collected and on these the absorbance at 280 nm, the conductivity, and the [^{3}H]UMP incorporation was determined.

Chromatography of both wild-type and mutant (α-am^r) soluble cell extracts showed multiple peaks of RNA polymerase. The pattern of elution with the ammonium sulfate gradient is shown in Fig. 1.

Fractions with enzymatic activity were pooled in two groups as shown in Fig. 1. Several characteristics of pool II have been determined (Table 2). Optimum activity was attained at 1 mM manganous chloride and 100 mM ammonium sulfate. Under these conditions, the sensitivity of wild-type and mutant enzyme to inhibition by α-amanitine was determined. Wild-type enzyme is inhibited by 70% by α-amanitine, whereas the mutant is highly resistant (Fig. 2). Since the inhibition by α-amanitine of the wild-type RNA polymerase was not complete, we tested the possibility of the presence in pool II of mitochondrial RNA polymerase [form III, see Jacob (16)]. Mitochondrial RNA polymerase is resistant to α-amanitine (13) but is sensitive to rifampicin (17). As shown in Table 2, rifampicin inhibits by 30% the RNA polymerase activity of wild-type pool II. This suggests that the fraction of wild-type activity resistant to α-amanitine (Fig. 2 and Table 2) represents mitochondrial RNA polymerase contamination. Since rifampicin inhibits α-am^r mutant pool II by approximately the same extent, we infer that also this pool contains mitochondrial RNA polymerase.

Mutant BHK-T6-G$_1$, therefore, appears to have an active, α-amanitine-resistant RNA polymerase II. The possibility that α-amanitine resistance is due to the complete absence of the normally α-amanitine-sensitive RNA polymerase molecules is excluded also by the stimulation of pool II activity by ammonium sulfate (Fig. 3).

The nucleolar (form I) RNA polymerase activity, assayed in pool I of the DEAE-Sephadex fractionation, was unaffected by 2.5 μg/ml of α-amanitine (data not shown) and its

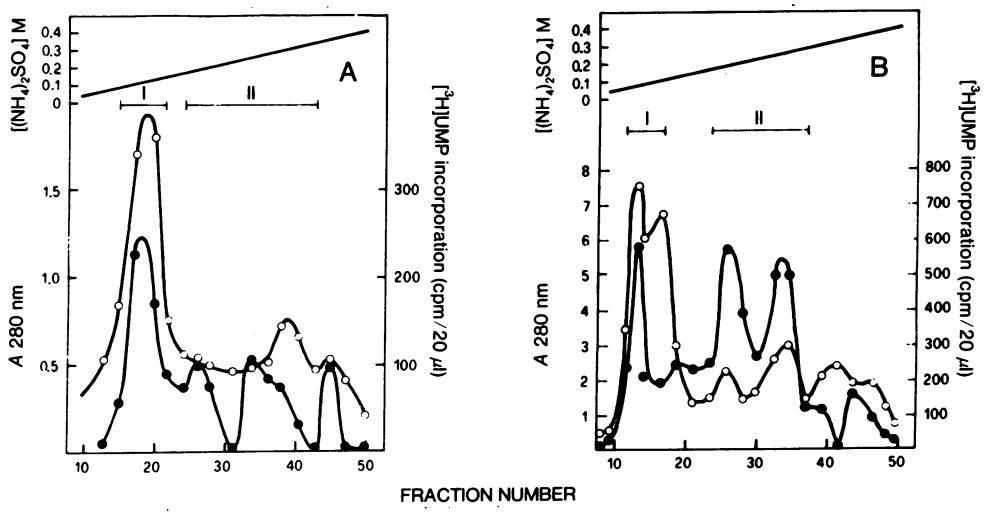

FIG. 1. DEAE-Sephadex elution profile of RNA polymerase activities of BHK-T6 and BHK-T6-G_1 cells. (A) α-am^s BHK-T6 cell extract. (B) α-am^r BHK-T6-G_1 cell extract. O, Absorbance at 280 nm; ●, incorporation of [^{3}H]UMP. In both cases, only the ammonium sulfate elution of the chromatography is shown. Horizontal bars represent the fractions pooled for subsequent analysis.

activity was not stimulated by ammonium sulfate in either α-am^s or α-am^r extracts (Fig. 3).

(d) Dominance Test of BHK-T6-G_1 α-am^r Mutation. To test whether the α-am^r allele is dominant over α-am^s, somatic cell hybrids were obtained by spontaneous fusion between hamster BHK-T6-G_1 and mouse 3T3-TK$^-$ cells by using the Littlefield hypoxanthine–aminopterin–thymidine (HAT) selecting system (18). Forty independent hybrid clones were selected and tested for growth in the presence of 2 μg/ml of α-amanitine and 0.5 μg/ml of amphotericin B. The dose of amphotericin B was lowered because of the high sensitivity of the 3T3 parent cells to this drug (10). All the 40 isolated hybrid clones had an equal efficiency of plating in the presence or absence of α-amanitine. Twenty control hybrids between BHK-T6 and 3T3-TK$^-$ cells were all α-amanitine-sensitive.

(e) Karyotype of Hybrid Lines. Mouse 3T3-TK$^-$ cells have a mean chromosome number of 65, with approximately 60 telocentric and five acrocentric chromosomes (9), whereas hamster BHK-T6-G_1 cells have a mean chromosome number of 44, with approximately 38 metacentric and six acro- or telocentric chromosomes. This difference makes it easy to analyze the hybrid karyotype as well as to determine the ratio of parental chromosomes.

From the hybrids mentioned before (section d) eight clones of BHK-T6-G_1 × 3T3-TK$^-$ (clones 01–14) and three clones of BHK-T6 × 3T3-TK$^-$ (clones 58–65) were analyzed for their chromosomal composition right after the first mass culture. Results of this test are reported in Table 3. In this table the test of sensitivity towards polyoma growth is also reported.

Since it is known that 3T3 parental chromosomes are easily lost in hamster–mouse hybrids, cells were frozen to assure further work on characterized lines. Two hybrid lines of each type were subcultured for a further 100 generations and retested for susceptibility to polyoma: all of them had lost susceptibility to viral growth, and concomitantly had a lower proportion of telocentric chromosomes (Table 3).

(f) Growth of Polyoma on Hybrid Clones in the Presence of α-Amanitine. As previously mentioned, hybrids obtained from fusion of BHK-T6 or BHK-T6-G_1 with 3T3-TK$^-$ cells were susceptible to polyoma; therefore, we infected cells from three α-amanitine-resistant and three sensitive hybrids at MOI of 0.2 and 10 in the absence of or in the presence of α-amanitine added 2 hr before infection or right after infec-

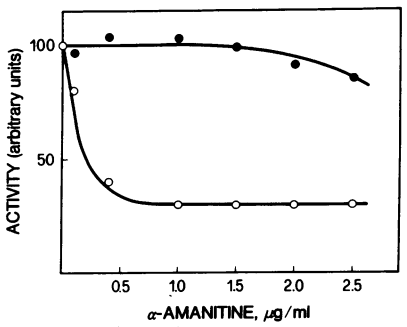

FIG. 2. Effect of α-amanitine on the RNA polymerase activity from pooled fractions (pool II) from the column chromatography of Fig. 1. Enzyme activity was determined at 1 mM $MnCl_2$ and 100 mM $(NH_4)_2SO_4$. O, Enzyme from BHK-T6 cells; ●, enzyme from BHK-T6-G_1 α-am^r cells.

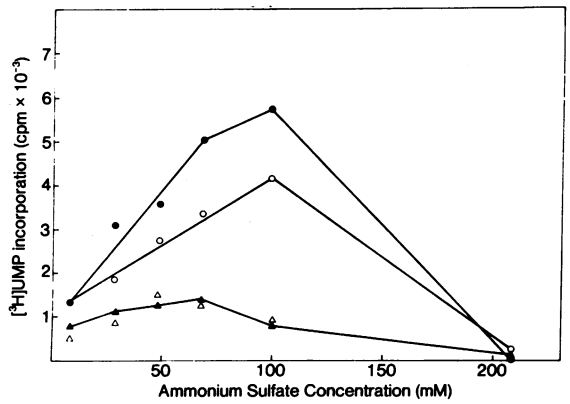

FIG. 3. Effect of ammonium sulfate concentration on RNA polymerase activity of pools I and II of Fig. 1. Assays were carried out at 1 mM $MnCl_2$. △, BHK-T6, pool I; ▲, BHK-T6-G_1, pool I; O, BHK-T6, pool II; ●, BHK-T6-G_1, pool II.

TABLE 3. *Chromosome composition, sensitivity to α-amanitine, and susceptibility to polyoma of BHK-T6 × 3T3-TK⁻ hybrid clones*

Hybrid clone no.*	α-Amanitine sensitivity† BHK parent	α-Amanitine sensitivity† hybrid	Chromosome constitution,‡ telocentric/total	Polyoma growth§
01	R	R	0.55	+
03	R	R	0.56	+
04	R	R	0.68	+
06	R	R	0.52	+
07	R	R	0.53	+
09	R	R	0.62	+
10	R	R	0.64	+
14	R	R	0.56	+
58	S	S	0.58	+
60	S	S	0.60	+
65	S	S	0.55	+
101	R	R	0.28	−
109	R	R	0.31	−
158	S	S	0.30	−
160	S	S	0.28	−

* The four hybrid lines with a number higher than 100 are the clones 01, 09, 58, and 60, respectively, grown for approximately 100 generations without hypoxanthine–aminopterin–thymidine selection.

† R = resistant, S = sensitive, at 2 μg/ml of α-amanitine and 0.5 μg/ml of amphotericin B.

‡ Determined as described in ref. 9; the data represent the average of at least five karyotypes. Telocentric chromosomes are mostly of mouse origin.

§ Test of polyoma growth was carried out at MOI of 10. The sign + represents a titer higher than that of the inoculum, −, a titer lower than that of the inoculum.

tion. Results reported in Table 4 show that polyoma grows in BHK-T6-G_1 × 3T3-TK⁻ hybrids (clones 01, 09, and 10) with the same efficiency in the presence and absence of α-amanitine. Very different are the results obtained with hybrid of BHK-T6 × 3T3-TK⁻ (clones 58, 60, and 65), since in these cells α-amanitine strongly inhibits polyoma growth, and the residual virus titrated could have originated from non-adsorbed or de-adsorbed infecting virions. Moreover, α-amanitine completely prevents polyoma growth in the 3T3 parental line. Addition to cells of α-amanitine 2 hr before infection or right after infection did not alter in any case the results, thus demonstrating that adsorption and penetration of α-amanitine is sufficiently rapid to express its real action. Burst size has been in some cases relatively low, in agreement with previously reported results of polyoma growth on hybrid cells (1). We also show that sensitivity of polyoma growth on 3T3-TK⁻ cells is not altered if amphotericin B is omitted during α-amanitine treatment. Since we have shown (Table 3) that hybrids that had extensively lost 3T3 chromosomes had also lost polyoma susceptibility but still remained resistant to α-amanitine, we can also conclude that the alteration of the RNA polymerase that gives rise to the phenotype α-am^r is not directly responsible for permissiveness in the hybrids analyzed.

DISCUSSION

α-Amanitine has been shown to be a potent inhibitor of RNA synthesis (4), acting on the nucleoplasmic form of RNA polymerase (form II) (13). It exerts its action at the level of chain elongation (19) by binding to the enzyme and not to DNA (20); in fact, hamster cells with α-amanitine-resistant RNA polymerase can be isolated (6). We have shown that the α-amanitine-resistant BHK-T6-G_1 mutant is altered in its RNA polymerase and that this mutation is dominant over the sensitive allele in hybrids with 3T3-TK⁻ mouse cells. Despite the multiplicity of the RNA polymerases and their lability during purification procedures, our data show that the mutant BHK-T6-G_1 has an α-amanitine-resistant RNA polymerase II. The assignment of the mutation to RNA polymerase II is based on: (a) in the mutant extracts, an α-amanitine-resistant RNA polymerase activity is eluted from DEAE-Sephadex at a salt concentration characteristic of

TABLE 4. *Yields of one-cycle growth of polyoma in hybrid (Hy) lines and parental 3T3-TK⁻ cells*

Cell line	Resistance to α-amanitine*	MOI	Viral titer(10^5 PFU/ml)† −α-Amanitine (a)	+α-Amanitine‡ 2 hr before infection (b)	+α-Amanitine‡ at infection	Ratio a/b
Hy 01	R	0.2	5.6	6.6	4.7	0.84
		10	31	27		1.14
Hy 09	R	0.2	5.6	6.6	4.9	0.84
		10	82	170		0.48
Hy 10	R	0.2	9.0	11.0	9.0	0.81
		10	110	130		0.84
Hy 58	S	0.2	36	0.45	0.14	80.0
		10	340	0.40		850
Hy 60	S	0.2	92	2.5	3.4	36.8
		10	340	10		34.0
Hy 65	S	0.2	30	1.6	1.8	18.7
		10	120	2.0		60.0
3T3-TK⁻	S	10	120	0.4		300
		10§	110	0.41		268

* As in Table 3.

† From 4 × 10^5 cells per plate with 5 ml of medium.

‡ In the presence of 2 μg/ml of α-amanitine and 0.5 μg/ml of amphotericin B.

§ Amphotericin B omitted.

RNA polymerase II. In the parental extracts, at that position, an α-amanitine-sensitive activity is found; (b) this activity, regardless of its sensitivity or resistance to α-amanitine, is stimulated by ammonium sulfate; (c) another α-amanitine-resistant activity, this one insensitive to ammonium sulfate, is eluted at the position characteristic of RNA polymerase I in the mutant and parental extracts; (d) DEAE-Sephadex elution profiles (Fig. 1A and B), despite some differences in peak heights and widths, show the same number of peaks of RNA polymerase activity in the parental and mutant extracts. The assignment of the mutation to an altered form of RNA polymerase II justifies the α-amanitine-resistant phenotype of the mutant cell line and its dominant behavior in somatic cell hybrids.

Transcription of the viral genome in transformed cells has been subjected to intensive research, especially with SV40. Martin and Axelrod (21), on the basis of saturation hybridization analysis of labelled RNA from SV40-transformed cells, suggested that all the sequences of RNA present in lytic infection could be found in some lines of transformed cells and that control of expression of viral genes could, therefore, be post-transcriptional. However, the introduction of more sophisticated techniques has allowed Ozanne *et al.* (22) to measure the percentages of early and late strands of SV40 DNA that are transcribed in different transformed cells. These authors have shown that in many SV40-transformed lines more "early" strand is transcribed than is found as stable message in lytic infection. A very short segment of the "late" strand is transcribed, and only in some lines.

This difference in the transcription pattern could be accounted for by a difference in the transcription mechanism or in the post-transcriptional processing of nuclear RNA, as proposed by Aloni (23) for lytic infection. The first possibility might be due to differences in the host RNA polymerase in correctly transcribing the viral genome for the lytic cycle. Our results that in the hybrids between BHK α-am^r and 3T3 α-am^s cells polyoma can grow with equal efficiency in the presence and absence of α-amanitine indicate that RNA polymerase II of the hamster, the nonpermissive cell line, is capable of transcribing correctly the viral genome. However, since it is not yet known how α-amanitine binds to the polymerase, we cannot exclude that in hybrid cells a hybrid RNA polymerase II can be formed with subunits of both parental enzymes.

The fact that hybrids that lost 3T3 chromosomes after prolonged culture also lost the susceptibility to polyoma but not the α-am^r phenotype excludes the possibility that a pleiotropic effect of the α-am^r mutation is responsible for the susceptibility of hybrids to polyoma. Therefore, the control of polyoma growth in different cell lines seems to lie at a level different from the activity of the host RNA polymerase. Of course, this does not exclude that the difference between permissive and nonpermissive cells can lie in specific factors acting on the RNA polymerase.

We are grateful to Dr. G. Marin for reviewing the manuscript and Mrs. M. Terracciano for technical help.

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