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The *In Vitro* Synthesis of Avian Myeloblastosis Viral RNA Sequences

(RNA synthesis/avian myeloblastosis virus/chromatin)

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ABSTRACT **Isolated nuclei, prepared from myeloblasts of chicks infected with avian myeloblastosis virus, synthesize RNA sequences present in avian myeloblastosis viral RNA. These sequences are also formed during transcription of chromatin, isolated from myeloblasts, by DNA-dependent RNA polymerases purified from *Escherichia coli* or calf thymus. In the latter case, transcription is α-amanitin sensitive. Formation of hybrids between RNA and avian myeloblastosis virus DNA probes has been monitored by the combined use of ribonucleases A, T_1, and H, and ribonucleases specific for single strands.**

RNA tumor viruses replicate via an undefined pathway dependent on both DNA synthesis and DNA-dependent RNA synthesis. The mechanisms involved in replication were partly resolved with the discovery by Baltimore and Mitzutani and Temin of an RNA-dependent dNTP incorporating system within RNA tumor virions. It is now well established that the genetic information required for production of RNA tumor viruses can exist in a cryptic state in animal cells and that virus formation can be induced by a variety of agents (*cf* 1, 2). A number of workers have also demonstrated that sequences complementary to the RNA genome of RNA tumor viruses are present in DNA isolated from various animal cells (1, 3–9). These observations suggest that replication of RNA tumor viruses may proceed via a pathway which includes integration of viral genetic information in the form of DNA in the host chromosome; this integrated information could be released in the form of RNA. With this hypothesis in mind, we examined nuclei isolated from myeloblasts of avian myeloblastosis virus (AMV) infected birds for their ability to synthesize RNA containing AMV RNA sequences; as presented in this communication, such sequences were detected. These observations have been extended to RNA formed during transcription of chromatin isolated from myeloblasts with highly purified DNA-dependent RNA polymerase of *Escherichia coli* or RNA polymerase B isolated from calf thymus.

MATERIALS AND METHODS

Synthesis and Isolation of DNA Complementary to AMV RNA. Reaction mixtures (1 ml) containing 50 mM Tris·HCl, pH 8.0 (at 37°), 10 mM $MgCl_2$, 5 mM dithioerythritol, 2 mM potassium phosphate, pH 8.0, 0.2 mM each of dATP, dGTP, and dCTP, 0.01–0.04 mM [^{3}H]dTTP (1 to 10 $\times$ 10^3 cpm/pmole), 5 mM phospho*enol*pyruvate, 0.1 unit of pyruvate kinase, 0.02% NP-40 detergent, 100 μg of actinomycin D, and 2 mg of AMV were incubated for 2–4 hr at 37°. Reactions were terminated by the addition of ethylenediaminetetraacetate (EDTA) (50 mM) and sodium dodecyl sulfate (1%), extracted with phenol, precipitated with ethanol, dissolved in 0.3 M NaOH, and incubated 10–16 hr at 37°. Low-molecular-weight material was removed by gel filtration through Sephadex G-50. The [^{3}H]DNA product was 98% single-stranded as determined by its sensitivity to *Neurospora* and S_1 nucleases, and its elution profile from hydroxylapatite. The product size, based on sedimentation in neutral sucrose gradients, was 4–6S as previously noted by others (*cf.* 2).

Isolation of 35S RNA from AMV. All glassware and solutions used in this procedure were sterile. AMV was concentrated from plasma by centrifugation at 80,000 $\times$ *g* for 30 min. The pellet was suspended in buffer (STE) containing 20 mM Tris·HCl, pH 8.0, 0.1 M NaCl, and 1 mM EDTA; 50 μg/ml of proteinase K and sodium dodecyl sulfate (final concentration of 0.5%) were added and the mixture was incubated at 23° for 30 min on a slow rotary mixer. The solution was extracted twice with phenol, precipitated with ethanol, dried, and redissolved in STE containing 0.1% sodium dodecyl sulfate. 70S RNA was separated from total RNA by centrifugation through a 10–30% sucrose gradient (containing STE and 0.1% sodium dodecyl sulfate) in an SW 50.1 rotor for 60 min at 48,000 rpm. [^{14}C]RNA markers (4S, 16S, and 23S) were centrifuged simultaneously in another gradient. Carrier yeast tRNA was added to the pooled 70S RNA, and the mixture was precipitated and collected as described above. The RNA dissolved in STE was then heat-denatured at 80° for 5 min, rapidly cooled on ice, and immediately centrifuged through a 10–30% sucrose gradient for 90 min at 60,000 rpm in an SW 65 rotor. Low-molecular-weight material was removed from the pooled 35S RNA by filtration through Sephadex G-50.

Enzymes. Neurospora nuclease was purified according to the method of Rabin and Fraser (10). RNase H from *E. coli* (11) was purified by Dr. I. Berkower of this Department. DNA-dependent RNA polymerase B (α-amanitin sensitive) was purified from calf thymus by the procedure of Kedinger and Chambon (12) through glycerol gradient centrifugation. Such enzyme fractions incorporated 30 nmole of UMP per mg of protein in 10 min at 37° at pH 8.0 using denatured salmon sperm DNA as template. DNA-dependent RNA polymerase from *E. coli* (holoenzyme) (13) was prepared by L. Yarbrough of this Department.

Isolation of Myeloblast Nuclei. Suspensions of myeloblasts stored at −60° were thawed, washed with Earle's medium

Abbreviations: AMV, avian myeloblastosis virus; EDTA, ethylenediaminetetraacetate.

(14) and collected by centrifugation at 2000 × *g* for 2 min. Nuclei isolated in the presence of Triton X-100 (15) were suspended in 20% glycerol containing 4 mM $MgCl_2$, 10 mM Tris·HCl, pH 7.9, and 5 mM dithioerythritol. Nuclei preparations were then frozen in liquid N_2 and stored at −60°. Purity and intactness of nuclei were monitored by phase microscopy.

Isolation of Chromatin and DNA from Myeloblast Nuclei. Chromatin was isolated from myeloblast nuclei as described by Huang and Huang (16) for the isolation of chromatin from chick-embryo nuclei with the following modifications: the saline–EDTA washes were omitted, the 0.5 M Tris·HCl wash was repeated once, and the 1 mM Tris·HCl wash was also omitted. After dissolving the chromatin in water, pH 8.0 (adjusted with NH_4OH), the solution was made 0.14 M and 0.01 M with respect to NaCl and Tris·HCl, pH 8.0, respectively, and incubated at 0° with gentle stirring for 30 min. Chromatin was collected by centrifugation, redissolved in H_2O, pH 8.0, and the NaCl wash was repeated. The chromatin suspension (in water) was then adjusted to 0.01 M Tris·HCl, pH 8.0, and centrifuged through 1.7 M sucrose as described by Bonner *et al.* (17). The chromatin pellet was redissolved in 2 volumes of H_2O and dialyzed for 12 hr against H_2O, pH 8.0. Chromatin suspensions were stored at 0°, and used within 2 weeks of preparation.

DNA from myeloblast nuclei was prepared by the procedure described by Gross-Bellard *et al.* (18).

Synthesis of [^{32}P]RNA with Myeloblast Nuclei. Reaction mixtures (0.1–0.5 ml) containing 40 mM Tris·HCl, pH 8.0, 4 mM dithioerythritol, 0.1 M $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 4 mM phospho*enol*pyruvate, pyruvate kinase (3 units/ml), 2 mM sodium phosphate, pH 8.0, 0.1 mM each of ATP, CTP, and GTP, 5–20 μM [α-^{32}P]UTP (5 to 20 × 10^3 cpm/pmole) and myeloblast nuclei (2–5 mg of protein per ml) were incubated for 60 min at 25°. The nuclei were then lysed by adding NaCl (final concentration 0.2 M), and the mixtures were digested with DNase I (0.1 mg/ml) for 30 min at 37°. RNA was isolated as described below.

Synthesis of [^{32}P]RNA with Myeloblast Chromatin and RNA Polymerase. Reaction mixtures (0.5 ml) containing 20 mM Tris·HCl, pH 8.0, 10 mM dithioerythritol, 2 mM $MnCl_2$, 0.6 mM each of ATP, GTP, and CTP, 0.1 M $(NH_4)_2SO_3$, 3 A_{260} units of myeloblast chromatin, 45–65 μg of calf-thymus RNA polymerase II or 1–2 μg of *E. coli* RNA polymerase and 25 μM [α-^{32}P]UTP (1 to 2 × 10^4 cpm/pmole) were incubated for 60 min at 38°. The samples were placed on ice and RNA was isolated as described below.

Isolation of [^{32}P]RNA Products. Pooled reaction mixtures were extracted twice with an equal volume of phenol saturated with 0.1 M NaCl, 0.02 M Tris·HCl, pH 8.0. Nucleic acids precipitated with 2 volumes of cold ethanol were collected by centrifugation, and dried *in vacuo*. The precipitate was dissolved in 0.5 ml of a solution containing 0.1 M NaCl, 10 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, and was incubated with 25 μg of DNase at 38° for 60 min. The phenol–extracted product was passed through a column of Sephadex G-50 previously equilibrated with 20 mM Tris·HCl, pH 8.0, 20 mM NaCl, and 2 mM EDTA. The radioactive product, which eluted in the void volume, was dialyzed against 1 mM Tris·HCl, pH 8.0, and concentrated approximately 10 to 20-fold *in vacuo*.

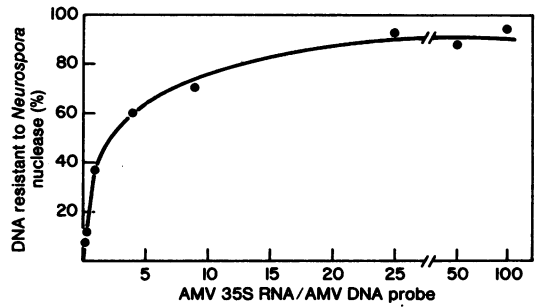

FIG. 1. Hybridization of AMV 35S RNA with AMV DNA probe. Various amounts of 35S AMV RNA were hybridized with AMV [^{3}H]DNA probe at $C_{R+D} \times t$ greater than 10^{-1} (C_{R+D} is the total concentration of RNA and DNA in moles of nucleotide/liter; t is sec). Samples were treated with *Neurospora* nuclease for 40 min at 37°, and acid-insoluble material remaining was determined as described in *Materials and Methods*. DNA in hybrid structure is defined as *Neurospora* nuclease resistant material. The results have been corrected for a background resistance of 1.5%. The RNA to DNA ratio is a molar ratio based on the absorbance at 260 nm of RNA; the amount of DNA added was calculated from the known specific activity of the labeled nucleotides used in its formation.

DNA·RNA Hybridization. [^{3}H]DNA probe and RNA transcript products were hybridized at 68° in 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, and 0.3 M NaCl in volumes ranging between 0.01 and 0.2 ml. Incubation times and concentrations of nucleic acid used varied in each experiment.

Isolation of DNA·RNA Hybrids. Isopycnic centrifugation of the radioactive products was as previously described (19). Material which sedimented in the hybrid region (ρ = 1.55 g/ml) was dialyzed against 10 mM Tris·HCl, pH 8.0, 1 mM EDTA for 3 hr with three changes of dialyzing medium.

Nuclease Treatment—RNase A and T_1.* Reaction mixtures (0.05–0.5 ml) containing 0.3–50 pmole of nucleic acid nucleotides, 20 mM Tris·HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml of RNase A, and 50 units/ml of RNase T_1 were incubated for 30 min at 37° and 50 μg of salmon sperm DNA, 0.1 ml of 0.1 M sodium pyrophosphate, and 2 ml of 10% trichloroacetic acid were added. Acid-insoluble material was collected by filtration on Whatman GF/C discs, washed three times with 5% trichloroacetic acid and once with ethanol. The filters were dried and their radioactivity measured in a scintillation spectrometer.

Neurospora Nuclease. Reaction conditions were similar to those described for RNases A and T_1 with the following exceptions: 10 mM $MgCl_2$ was present, the concentration of NaCl was 30 mM or less, and 1 unit/ml of *Neurospora* nuclease replaced the other nucleases.

RNase H. Reaction conditions were similar to those described for RNases A and T_1 with the following exceptions: 10 mM $MgCl_2$, 50 μg/ml of bovine serum albumin and 2 mM dithioerythritol were also included, and 20–40 units/ml of *E. coli* RNase H replaced the other nucleases.

* One unit of RNase T_1 and *Neurospora* nuclease converted 100 nmoles of nucleotides to an acid-soluble form in 30 min at 37°; one unit of *E. coli* RNase H released 1 nmole of acid-soluble nucleotide in 30 min at 37° from [^{3}H]poly(A) in the presence of poly(dT).

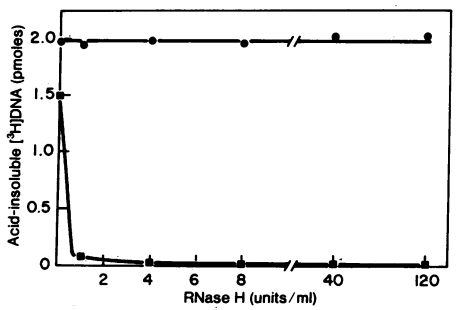

FIG. 2. The effect of RNase H on hybrids of 35S RNA and [^{3}H]DNA. AMV [^{3}H]DNA probe and 35S AMV RNA were hybridized as described in Fig. 1 at RNA:DNA of 15:1. Aliquots of the hybridization mixture were incubated with the indicated amounts of RNase H and *Neurospora* nuclease (■) or RNase H alone (●) as described in *Materials and Methods*.

Protein Determinations. Protein concentrations were determined spectrophotometrically (20).

RESULTS

Characterization of DNA Probe. DNA synthesized by AMV made permeable by detergent was used as aprobe for AMV RNA. The hybridization of this DNA with viral RNA is shown in Fig. 1. Since 35S components of 70S RNA contain sequences of viral origin (21, 22), the amount of DNA probe that formed hybrid structures with 35S AMV RNA was measured. Annealing of the DNA with a 20-fold excess of 35S RNA protected more than 90% of the probe from digestion by *Neurospora* nuclease. Thus, nearly all of the sequences in this DNA† are complementary to 35S RNA. The shape of the saturation curve suggests an unequal representation of viral sequences in the DNA product. Similar findings have been previously reported for other RNA oncogenic viruses (*cf.* 2).

The Use of RNase H for Hybrid Determinations. Several procedures were used to quantitate RNA·DNA hybrid structures; these included isopycnic centrifugation in Cs_2SO_4, hydroxylapatite chromatography, and measurement of sensitivity of products to different nucleases. In particular, we utilized both a nuclease specific for single-stranded polynucleotides and RNase H to measure RNA·DNA hybrids. *E. coli* RNase H is an endonuclease that degrades RNA solely in an RNA·DNA hybrid structure. Thus, as shown in Fig. 2, AMV [^{3}H]DNA probe hybridized to an excess of 35S RNA converted 80% of the DNA resistant to *Neurospora* nuclease. Addition of RNase H‡ rendered this resistant DNA susceptible to *Neurospora* nuclease. Since RNase H lacked DNase activity, these results indicate that RNase H had liberated single-stranded DNA which was attacked by *Neurospora* nuclease.

TABLE 1. *Hybridization of nuclear RNA transcripts to AMV DNA probe*

Hybridization mixture	pmole of [^{32}P]RNA remaining after treatment with RNases: A + T_1 (*a*)	A + T_1 + H (*b*)	Difference (*a* − *b*), pmole	[^{32}P]RNA in RNA·DNA hybrid, %
A	33.1	5.2	27.9	2.1
B	71.0	20.0	51.0	0.64
C	55.0	18.3	36.7	0.46
D	20	20	0	0

The conditions for synthesis and isolation of [^{32}P]RNA from myeloblast nuclei were as described in *Materials and Methods*. The specific activity of the isolated RNA was 20 cpm/pmole. Hybridization mixtures containing the following amounts of viral [^{3}H]DNA probe and [^{32}P]RNA (in nmole) in a total volume of 0.1 ml were incubated at 68° for 15 hr: (*A*) 1.35 RNA, 0.6 DNA; (*B*) 8 RNA, 0.6 DNA; (*C*) 8 RNA, 0.1 DNA; (*D*) 8 RNA. Duplicate aliquots of hybridization mixtures were treated with either RNases A and T_1, or RNases A, T_1, and H. RNA in RNA·DNA hybrid structure, defined as material resistant to RNase A and T_1 but sensitive to RNases A + T_1 + RNase H, is reported under the heading *Difference*.

Viral RNA Synthesis in Isolated Myeloblast Nuclei: Requirements and Detection of AMV Specific RNA. RNA synthesis in isolated nuclei maximally occurred in the presence of a divalent cation (Mn^{++} was 1.5-fold more effective than Mg^{++}), 4 rNTPs, and relatively high concentrations (100 mM) of $(NH_4)_2SO_4$; RNA synthesis was inhibited by 5 μg/ml of actinomycin D. RNA products formed by nuclei were completely sensitive to alkali and more than 99% sensitive to RNase A. α-Amanitin, a specific inhibitor of nucleoplasmic RNA polymerase (12), inhibited nucleotide incorporation 80%.

The amount of AMV RNA in newly synthesized products was determined by annealing isolated nuclear RNA with AMV[^{3}H]DNA probe at different ratios of DNA to RNA. As seen in Table 1, from 0.5 to 2% of the total transcript products were recovered in hybrid structure. In several other experiments (not shown) the amount of hybridizable RNA varied between 1 and 2%. It should be noted that although a preformed pool containing viral RNA is present in nuclei, this procedure specifically detects newly formed radioactive RNA§. RNA synthesis catalyzed by liver nuclei from uninfected chicks did not yield detectable AMV RNA among the transcript products (not shown). The addition of α-amanitin inhibited production of viral RNA.

RNA Synthesis from Myeloblast Chromatin with Purified RNA Polymerase: Characteristics and Detection of AMV RNA. It recently has been shown that globin RNA can be transcribed *in vitro* from reticulocyte chromatin by *E. coli* RNA

† The interaction between DNA probe and 35S RNA was further characterized. Hybrid 35S RNA·DNA structures possessed a melting temperature, T_m, of 88.5° and melted sharply. This can be compared to a T_m of 90.5° found for double-stranded AMV DNA formed in the absence of actinomycin D. The latter measurements were made with double-stranded AMV DNA eluted from hydroxylapatite columns between 0.15 and 0.3 M sodium phosphate buffer, pH 6.8, at 60°.

‡ RNase H (6 units) assayed as previously described (15) released no detectable (<0.01 nmole) material from [^{3}H]poly(C) or [^{3}H]poly(I) (200 nmoles of either), or [^{3}H]poly(I) in the presence of poly(C).

§ These experiments do not distinguish between *de novo* initiated RNA chains or preexisting RNA chains which have been elongated. However, in transcription studies with chromatin and calf-thymus RNA polymerase B, evidence of *de novo* chain formation has been obtained.

TABLE 2. *Hybridization of AMV DNA probe with RNA formed from chromatin by E. coli and calf-thymus RNA polymerase*

Method used for hybrid determination	Input [^{32}P]RNA in hybrid structure, %		
	RNA formed with RNA polymerase of		
	Calf thymus	*E. coli*	Control (RNA alone)
A	0.92	1.10	<0.02
B	0.87	0.90	<0.02
C	0.81	1.20	<0.01
D	0.84	0.63	<0.05

[^{32}P]RNA was isolated from reaction mixtures containing either *E. coli* RNA polymerase or calf thymus RNA polymerase B, myeloblast chromatin, and [α-^{32}P]UTP (10,000 cpm/pmole). Labeled products (0.8 or 1.6 nmole formed with calf thymus or *E. coli* RNA polymerase preparations, respectively) were hybridized with 300 pmole of AMV [^{3}H]DNA probe in a total volume of 0.2 ml; hybridization mixtures were incubated for 30 hr at 68° and [^{32}P]RNA present in hybrid structure was determined as follows: (*A*) After treatment with RNases A and T_1, acid-insoluble material was measured. (*B*) The material obtained in *A* was digested with RNase H and acid-insoluble material was determined. The results presented represent acid-soluble material formed. (*C*) Material resistant to *Neurospora* nuclease was isolated by isopycnic banding in Cs_2SO_4 (ρ = 1.55 g/ml) and was treated with RNases A and T_1. (*D*) Material isolated by isopycnic banding as in *C* was treated with RNase H. Control samples (RNA alone) represent hybridization carried out with 1.6 nmole of RNA formed with *E. coli* RNA polymerase in the absence of AMV DNA probe. Similar results were obtained with RNA formed with calf thymus RNA polymerase.

polymerase (23). The detection of significant amounts of AMV RNA in nuclear transcript products suggested that viral specific sequences might also be found in the RNA transcribed *in vitro* from myeloblast chromatin by an exogenous RNA polymerase. Since studies with myeloblast nuclei suggested that AMV-specific sequences were synthesized uniquely by the α-amanitin sensitive nucleoplasmic enzyme, the products synthesized from chromatin by the corresponding enzyme isolated from calf thymus were examined for these sequences. The requirements for RNA synthesis from myeloblast chromatin by calf-thymus RNA polymerase B were similar to those reported above for isolated nuclei except that Mn^{++} stimulated synthesis 10 to 20-fold more than Mg^{++}. The amount of virus-specific RNA in the transcript products was determined as shown in Table 2. Subsequent to annealing the isolated product with a 100-fold excess of the AMV DNA probe, approximately 1% of the total product was present in stable RNA·DNA hybrid structures. Similar results were obtained when *E. coli* RNA polymerase was substituted for the mammalian enzyme.

The validity of the techniques used to measure hybrid structures was supported by the following results. The material resistant to *Neurospora* nuclease banded at hybrid density (ρ = 1.55 g/ml) upon isopycnic centrifugation in Cs_2SO_4, but shifted to the density of DNA (ρ = 1.43 g/ml) after treatment with RNase H. Incubation of myeloblast chromatin transcript products under annealing conditions without AMV [^{3}H]DNA probe or annealing of RNA transcript products formed in reactions primed with calf-thymus DNA with the DNA probe

TABLE 3. *Template specificity of AMV RNA synthesis*

DNA template used	RNA polymerase used (μg of protein)	RNA synthesized, nmole	% input RNA hybridized to AMV DNA probe
Myeloblast chromatin (70 μg)	*E. coli* (1.0)	2.20	0.80
Myeloblast chromatin (70 μg)	Calf thymus (45)	1.33	0.85
Myeloblast native DNA (33 μg)	Calf thymus (90)	0.82	<0.05
Myeloblast denatured DNA (22 μg)	Calf thymus (60)	8.10	<0.03
Calf-thymus chromatin (80 μg)	Calf thymus (45)	1.10	<0.05

Reaction mixtures described in *Materials and Methods* with [α-^{32}P]UTP (10^4 cpm/pmole) were incubated for 60 min at 37°. The isolation and hybridization were carried out as described; RNA·DNA hybrid structures were measured as [^{32}P]RNA resistant to RNase A + T_1 but sensitive to RNase H. The concentration of DNA template was calculated assuming that 1 mg of material was equivalent to 20 absorbance units at 260 nm.

did not yield detectable RNA·DNA hybrid structures. In other experiments (Table 3) RNA synthesized from native or denatured DNA isolated from myeloblasts or from calf-thymus chromatin with calf-thymus RNA polymerase preparations did not yield detectable AMV RNA·DNA hybrids. Similar results were obtained when *E. coli* polymerase was used in place of calf-thymus RNA polymerase. The specificity of hybridization was tested in experiments in which various amounts of 35S AMV RNA were allowed to compete during annealing of the radioactive chromatin transcripts with AMV [^{3}H]DNA probe (Table 4). In the absence of 35S AMV RNA approximately equal amounts of transcript RNA and DNA were present in hybrid structure; however, at increasing levels of 35S AMV RNA the amount of DNA in these structures increased while that of transcript RNA decreased. These findings demonstrate that transcript products that hybridize with the viral DNA probe have sequences that coincide with those present in 35S AMV RNA.

DISCUSSION

Viral specific sequences should constitute a small fraction of the total RNA synthesized *in vitro*, since only 4–13 viral DNA equivalents are present in the genome of AMV-infected cells (3, 24). The hybridization assays described in this paper were designed to determine AMV RNA in the presence of a large excess of nonviral RNA. AMV-specific RNA sequences in *in vitro* transcripts were detected by hybridization with AMV-specific DNA synthesized by RNA-directed DNA polymerase in purified virions in the presence of actinomycin D. While different techniques were used to measure RNA·DNA hybrid structures, the most selective method that yielded reproducible results and low background values used a hybrid-specific RNase, RNase H, in conjunction with single-stranded nucleases (either RNases A and T_1, or *Neurospora* nuclease). RNA that hybridized with viral DNA probe was shown to be viral-specific by competition hybridization with 35S RNA isolated from heat denatured 70S AMV RNA. A possible diffi-

TABLE 4. *Competition hybridization between 35S RNA and RNA products formed from chromatin for AMV DNA probe*

Amount of 35S RNA present during hybridization, pmole	DNA in hybrid, %	[^{32}P]RNA in hybrid, %
0	2.1	1.70
100	28.4	0.90
1500	78.0	<0.03
3000	98.5	<0.03

Synthesis and isolation of [^{32}P]RNA from reaction mixtures containing calf-thymus RNA polymerase B, myeloblast chromatin, and [α-^{32}P]UTP (10^4 cpm/pmole) were described in *Materials and Methods*. Hybridization mixtures (0.2 ml) containing 150 pmole of AMV [^{3}H]DNA probe (1.2×10^3 cpm/pmole of nucleotides), 200 pmole of [^{32}P]RNA, and the indicated amounts of 35S AMV RNA were incubated 45 hr at 68°. DNA in hybrid structures was measured as DNA rendered sensitive to *Neurospora* nuclease (1.2 units/ml) by RNase H (40 units/ml). The background level of DNA resistant to *Neurospora* nuclease with RNase H was 1.2%. [^{32}P]RNA in hybrid structure was measured as [^{32}P]RNA resistant to a sequential degradation by *Neurospora* nuclease (1.2 units/ml) in 30 mM NaCl followed by RNase A and T_1 (50 μg/ml and 50 units/ml, respectively) in 100 mM NaCl, but which was made acid-soluble by RNase H (40 units/ml). No hybrid RNA was found in the control RNA annealed alone. The material resistant to RNase H after this treatment was between 0.5 and 1% of the total RNA.

culty with hybridization assays with DNA synthesized from virions is that RNA tumor viruses may contain small amounts of DNA or RNA of host origin (ref. 25; *cf.* 1, 20). Contaminating host-specific sequences contained in the DNA probe could alter the specificity of hybridization assays by converting host-specific RNA sequences into hybrid structures. This possibility seems remote in the experiments reported here since at least 90–95% of the viral probe DNA formed stable hybrid structures with 35S AMV RNA. The conclusion that the hybridization assay was detecting virus-specific RNA was further reinforced by negative results obtained with RNA transcribed in isolated liver nuclei from uninfected chickens.

The main purpose of these experiments was to quantitate the amount of viral-specific RNA transcribed *in vitro* from myeloblast chromatin by *E. coli* and calf-thymus RNA polymerases. Approximately 1% of the total products transcribed by the mammalian and bacterial polymerase formed stable RNA·DNA hybrid structures with the viral DNA probe. Since this figure agrees with the amount of viral-specific RNA found in nuclear transcript products, these results suggest that the procedure used to isolate chromatin had not severely altered its physical state. In agreement with the observations of Axel *et al.* (23) on globin RNA synthesis, AMV-specific RNA was not detected in products transcribed from native or denatured myeloblast DNA. A plausible explanation for the latter result is that removal of non-DNA elements from chromatin allowed transcription of a greater number of sequences, thus causing viral RNA to be diluted to undetectable levels. The amount of virus-specific RNA formed from chromatin can be calculated to be approximately 10^3-fold higher than would be expected from a random transcription of the few copies of integrated viral DNA. This amplification must result from a restriction in the transcription process. Since viral-specific sequences were amplified to the same extent by both eukaryotic and prokaryotic polymerase preparations, the restriction of transcription probably occurs at the level of chromatin under the present experimental conditions.

Note Added in Proof. While this paper was in press we learned that Rymo *et al.* (26) have obtained similar results with isolated nuclei from Rous sarcoma virus-infected cells.

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