Lai, C. J., Weisblum, B., Fahnestock, S. R., and Nomura, M. (1973), *J. Mol. Biol. 73* (in press).
Saito, T., Shimizu, M., and Mitsuhashi, S. (1971), *Ann. N. Y. Acad. Sci. 182*, 267.
Sanger, F., Brownlee, G. G., and Barrell, B. G. (1965), *J. Mol. Biol. 13*, 373.
Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry 2*, 697.
Weisblum, B., and Demohn, V. (1969), *J. Bacteriol. 98*, 447.
Weisblum, B., Siddhikol, C., Lai, C. J., and Demohn, V. (1971), *J. Bacteriol. 106*, 835.

Transcription of Ribonucleic Acid by the Ribonucleic Acid Directed Deoxyribonucleic Acid Polymerase of Rous Sarcoma Virus and Deoxyribonucleic Acid Polymerase I of *Escherichia coli*†

J. M. Taylor,* A. J. Faras, H. E. Varmus, H. M. Goodman, W. E. Levinson, and J. M. Bishop

ABSTRACT: The DNA synthesized *in vitro* by the purified RNA-directed DNA polymerase of Rous sarcoma virus (RSV), using tumor virus RNA as template, contains both single- and double-stranded molecules. Both forms are small and the double-stranded form is copied primarily from particular regions of the RNA. These same properties are found to apply to the DNA transcribed from poliovirus RNA. The primer $(dT)_{12-18}$ augments the response of the enzyme to RSV RNA, heat-denatured RSV RNA, and poliovirus RNA. The size of the DNA products is still small and the portion of the RNA template represented in double-stranded DNA is decreased. In transcription from RSV RNA, the use of $(dT)_{12-18}$ produces additional initiation sites on the template although DNA is not transcribed from regions other than those previously transcribed. DNA synthesis on 70S RSV RNA template is initiated by the formation of a phosphodiester bond between a 3′-terminal adenosine moiety and deoxyadenosine. Addition of $(dT)_{12-18}$ stimulates the overall synthesis of DNA and does not interfere with the formation of the above-mentioned bond. The DNA-dependent DNA polymerase I of *Escherichia coli* does not respond to naturally occurring RNA templates. However, addition of $(dT)_{12-18}$ to poliovirus RNA and RSV RNA, which possess poly(A) sequences, permits the synthesis of DNA limited to poly(dA) and poly(dT) rather than heteropolymer.

The RNA-directed DNA polymerase of RNA tumor viruses has been purified in several laboratories (Kacian *et al.*, 1971; Duesberg *et al.*, 1971a; Hurwitz and Leis, 1972; Faras *et al.*, 1972). These preparations are entirely dependent on the addition of exogenous template and are sufficiently free of ribonuclease activity to allow the use of ribonuclease-sensitive naturally occurring RNA templates. They respond to DNA and to synthetic homopolymers (such as poly(dA·dT) and poly(A)·oligo(dT)), but display a preference for 70S oncornavirus RNA as template among the naturally occurring RNAs tested (Duesberg *et al.*, 1971a; Faras *et al.*, 1972). The DNA transcribed from 70S RNA has been generally found to possess the following properties. First, the DNA molecules are small (4–10 S) relative to the size of the oncornavirus genome (reviewed by Temin and Baltimore, 1973). Secondly, transcription into ds-DNA[1] occurs predominantly from very limited regions of the genome (Gelb *et al.*, 1971; Varmus *et al.*, 1971; Taylor *et al.*, 1972). These findings led us to investigate whether the same two properties of the DNA product apply to other situations of RNA-directed DNA synthesis. We have used the purified polymerase of Rous sarcoma virus (RSV) to study the transcription of RSV and poliovirus RNAs, with particular emphasis on the effects of an exogenously added primer molecule $(dT)_{12-18}$ (Duesberg *et al.*, 1971b). In addition, we have analyzed the nature of RNA-directed DNA synthesis by DNA polymerase I of *Escherichia coli*.

Materials and Methods

Reagents and Solutions. Deoxynucleoside triphosphates were from Calbiochem. [^{3}H]Deoxynucleoside triphosphates (5–24 Ci/mmol) were from Schwarz BioResearch, Inc. [α-^{32}P]-Nucleoside triphosphates (4–16 Ci/mmol) were from International Chemical and Nuclear Corp. $(dT)_{12-18}$ was from Collaborative Research, Inc.

† From the Departments of Microbiology and Biochemistry, University of California, San Francisco, California 94122. *Received July 3, 1972.* This investigation was supported by U. S. Public Health Service Grants AI 08864, CA 12380, CA 12705, AI 06862, and AI 00299, American Cancer Society Grant P-591, and Contract 71-2147 within the Special Virus-Cancer Program of the National Cancer Institute, the National Institutes of Health, and the Public Health Service. J. M. T. and H. E. V. acknowledge the support by Senior Dernham Fellowships (D-201 and D-164, respectively), of the American Cancer Society, California Division.

[1] Abbreviations used are: ds-DNA, double-stranded DNA; ss-DNA, single-strand DNA; RSV, Rous sarcoma virus; C_0t, concentration × time, expressed as (mol sec)/l.

TABLE I: Stimulation of DNA Synthesis by $(dT)_{12-18}$.[a]

RNA Template	Rel Incorporation	
	$-(dT)_{12-18}$	$+(dT)_{12-18}$
RSV	1.00	4.2
RSV (denatured)	0.04	2.8
Poliovirus	0.09	10.1

[a] Standard reactions of one hour were used and the incorporation of [^{3}H]TTP (at 8000 cpm/pmol) into acid-insoluble material is expressed relative to the incorporation observed with RSV RNA as template in the absence of added primer (0.5 pmol). The primer $(dT)_{12-18}$ was used at a concentration of 0.1 μg/ml. The templates were used at 2 μg/ml which is nonsaturating for the enzyme under the present conditions (Faras *et al.*, 1972).

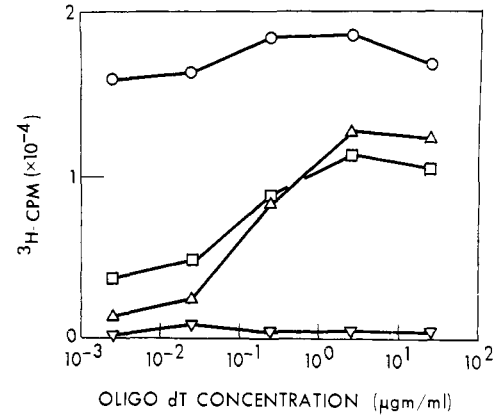

FIGURE 1: Stimulation of DNA synthesis by $(dT)_{12-18}$. Standard reaction mixtures (0.05 ml) containing 0.2 unit/ml of purified polymerase were incubated for 1 hr at 37°. The incorporation of [^{3}H]TTP into acid-insoluble material is shown as a function of $(dT)_{12-18}$ concentration, when the template is calf thymus DNA (○), RSV RNA (□), poliovirus RNA (△), or in the absence of template (▽). All templates were used at a concentration of 2 μg/ml, which has been shown to be nonsaturating for the polymerase under the present assay conditions (Taylor *et al.*, 1972).

Methods. We have previously described the purification of the RNA-directed DNA polymerase of Rous sarcoma virus (Faras *et al.*, 1972). One unit of polymerase is defined as that amount of activity which catalyzes the incorporation into acid-insoluble material of 1 nmol of [^{3}H]TTP in 2 hr at 37°, with calf thymus DNA (200 μg/ml) as template. The standard reaction mixture contains: 0.1 M Tris-HCl (pH 8.1), 0.01 M $MgCl_2$, 2% β-mercaptoethanol, three unlabeled deoxynucleoside triphosphates at 5×10^{-5} M each, a fourth labeled triphosphate at 5×10^{-6} M, template nucleic acid at 2 μg/ml, and purified polymerase at 0.2 unit/ml.

Most of the experimental procedures used have been documented elsewhere. The preparation of RSV 70S RNA, poliovirus 35S RNA, phage R17 27S RNA, and HeLa cell rRNA have been described by Faras *et al.* (1972), and the procedures of nearest-neighbor analysis, rate zonal centrifugation, and equilibrium centrifugation have been described by Taylor *et al.* (1972). The reassociation of dsDNA was performed in 0.4 M phosphate buffer and assessed by fractionation on hydroxylapatite. The kinetics of reassociation are expressed for standard conditions (Britten and Kohne, 1968; Varmus *et al.*, 1971). In certain experiments reassociation was also assessed by use of the single strand-specific nuclease of *Aspergillus oryzae* (Leong *et al.*, 1972). In such situations, the reassociation was performed in 0.6 M NaCl because of the inhibitory effect of phosphate buffer on the nuclease.

Results

Effect of Exogenous Primer Molecules on DNA Synthesis. The response of the purified RNA-directed DNA polymerase of RSV to certain naturally occurring RNA templates can be stimulated by the addition of $(dT)_{12-18}$ (Duesberg *et al.*, 1971b; Robert *et al.*, 1972). The extent of this stimulation depends on the concentration of $(dT)_{12-18}$ relative to that of template when the latter is kept at a fixed nonsaturating concentration (Figure 1). Such a stimulation of template response is observed with RSV and especially poliovirus RNA (Figure 1), but not at all with rRNA or phage R17 RNA (data not shown). $(dT)_{12-18}$ by itself does not act as a template nor will it stimulate the response of the polymerase to calf thymus DNA.

Five RNA-directed reactions were chosen for analysis: RSV RNA without and with $(dT)_{12-18}$, denatured RSV RNA with $(dT)_{12-18}$, and poliovirus RNA without and with $(dT)_{12-18}$. The amount of DNA synthesized by the polymerase in each of these reactions is summarized in Table I. As has been previously shown, denaturation of RSV RNA (by heating at 80° for 2 min in 0.01 M EDTA–0.02 M Tris-HCl, pH 7.4) reduces its ability to act as a template for the polymerase (Duesberg *et al.*, 1971b; Faras *et al.*, 1972), yet this same denatured RNA gives a good response in the presence of $(dT)_{12-18}$ (Duesberg *et al.*, 1971b; Table I).

Nearest-Neighbor Analysis of DNA Product. In each of the five reactions mentioned above, the DNA products are predominantly heteropolymers on the basis of nearest-neighbor analysis (Table II). It should be noted however that the DNA synthesized in the presence of $(dT)_{12-18}$ contains an amount of poly(dT). The amount of this homopolymer in the DNA product is not increased when the concentration of $(dT)_{12-18}$ in the reaction mixture is increased from 0.1 to 2.0 μg per ml (Table II).

Sedimentation Analysis of DNA Product. The DNAs transcribed from both RSV 70S RNA and poliovirus 35S RNA have approximately the same chain length following denaturation with alkali (Figure 2a,d). The presence of exogenous primer $(dT)_{12-18}$ during synthesis does not appreciably affect the size of the DNA product in either instance (Figure 2b,e). The DNA synthesized with denatured RSV RNA and exogenous primer is also small (Figure 2c).

Initiation of DNA Synthesis. Initiation of DNA synthesis by the oncornavirus polymerase occurs on the 3′-hydroxyl terminus of a *primer* molecule irrespective of whether the *template* is RNA or DNA (Smoler *et al.*, 1972). A polyribonucleotide serves as primer for synthesis directed by naturally occurring RNAs (Verma *et al.*, 1971; Leis and Hurwitz, 1972; Taylor *et al.*, 1972). We have identified the 3′-terminal ribonucleoside of the primer molecule for RSV 70S RNA-directed synthesis by transferring α-^{32}P to it from a deoxynucleoside triphosphate precursor. The enzymatic product from reactions using each of the [α-^{32}P]deoxynucleoside triphosphates was hydrolyzed with alkali, leaving ^{32}P on the 3′-terminal ribonucleoside of the primer molecule. The labeled ribonucleotide is then identified by electrophoresis

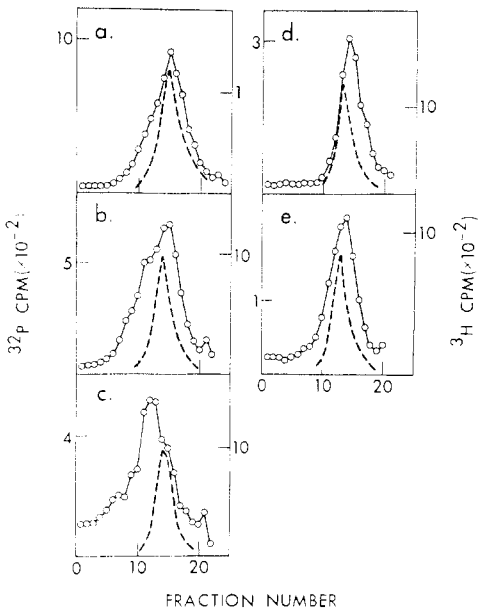

FIGURE 2: Velocity sedimentation of product DNA. Standard reactions were run for one hour, using [^{32}P]dCTP as the labeled nucleotide precursor. The products were then passed through a 40 × 0.9 cm column of Sephadex G-50 (coarse), concentrated by ethanol precipitation, boiled in 0.3 N alkali for 5 min (to remove any RNA and any secondary structure in the DNA product), neutralized, and subjected to velocity sedimentation analysis on 15-30% sucrose gradients (0.1 M NaCl–0.001 M EDTA–0.02 M Tris-HCl, pH 7.4), at 50,000 rpm for 15 hr at 4° in a SW-65 rotor. In each case 4S[^{3}H]-RNA was incorporated as a sedimentation marker (---). The [^{32}P]DNA products (○ ○) were synthesized under the following conditions of template (2 μg/ml) and primer (0.1 μg/ml): (a) template RSV RNA, no added primer; (b) RSV RNA plus primer $(dT)_{12-18}$; (c) denatured RSV RNA plus primer $(dT)_{12-18}$; (d) poliovirus RNA, no added primer; (e) poliovirus RNA plus primer $(dT)_{12-18}$.

(Table III). This procedure is similar to that employed by several other laboratories (Hurwitz and Leis, 1972; Flügel and Wells, 1972; Verma *et al.*, 1972a).

When [α-^{32}P]dATP is used as the labeled precursor 3.68% of the ^{32}P is found in Ap at the end of a 1-hr reaction (Table III). No ribonucleotides are labeled when any of the other [α-^{32}P]deoxyribonucleoside triphosphate are used. We have obtained qualitatively indentical results with the endogenous reaction of detergent-disrupted RSV (Table IV). It can thus be deduced that the average length of the DNA product to which one AMP is linked is (100/3.68) × 4 = 109 nucleotides. Direct measurements of the size of the product by gel electrophoresis indicate an average length of 80–100 nucleotides (unpublished observation). When $(dT)_{12-18}$ is used in the reaction, there is a reduction in the fraction of the DNA product to which AMP is attached. However, if this fraction is normalized to allow for the stimulation achieved by the addition of $(dT)_{12-18}$, one obtains a figure of 3.69%, comparable to the 3.68% obtained for the reaction performed without $(dT)_{12-18}$ (Table III). Thus, the introduction of $(dT)_{12-18}$ causes a stimulation of DNA synthesis but does not interfere with initiation of DNA synthesis at the intrinsic RNA primer whose 3′ terminus is adenosine.

When denatured RSV RNA is used as a template, the response is low unless $(dT)_{12-18}$ is added (Table I) and under such conditions covalent attachment of DNA product to RNA is not detected (Table III). Thus, after denaturation, the intrinsic primer RNA molecules are no longer available and DNA synthesis is initiated only at the 3′-OH of the $(dT)_{12-18}$.

TABLE II: Nearest-Neighbor Analysis of DNA Products.[a]

[α-^{32}P]-Nucleoside Triphosphate	RNA Template (2 μg/ml)	Exogenous Primer Concn (μg/ml)	Percentage of Label Transferred to			
			dCp	dAp	dGp	dTp
dCTP	RSV	0	23	22	30	25
	RSV	0.1	19	27	34	20
	RSV (denatured)	0.1	19	27	36	18
	Poliovirus	0	20	20	38	22
	Poliovirus	0.1	24	27	28	21
dATP	RSV	0	22	31	24	23
	RSV	0.1	19	42	19	20
	RSV (denatured)	0.1	21	38	20	21
	RSV (denatured)	2	36	35	19	10
	Poliovirus	0	21	29	26	24
	Poliovirus	0.1	26	35	21	18
dGTP	RSV	0	18	20	25	37
	RSV	0.1	18	21	23	38
	RSV (denatured)	0.1	19	25	24	32
	Poliovirus	0	14	24	27	35
	Poliovirus	0.1	8	27	23	42
dTTP	RSV	0	23	27	28	22
	RSV	0.1	13	18	14	54
	RSV	2	14	19	12	55
	RSV (denatured)	0.1	13	17	14	56
	RSV (denatured)	2	16	20	14	50
	Poliovirus	0	21	27	26	26
	Poliovirus	0.1	24	18	19	39

[a] DNA products were synthesized in standard reactions of one hour, with conditions of template, primer, and [α-^{32}P]-nucleoside triphosphate as indicated in the table. The products were treated with sodium dodecyl sulfate (0.1%), passed through a column of Sephadex G-50 to remove free nucleoside triphosphate and subsequently analyzed for nearest neighbors by methods previously described (Taylor *et al.*, 1972). A typical standard deviation for the percentages shown is 5.

Extent of Transcription from RNA into DNA. The extent of transcription of RNA into ds-DNA can be determined by denaturing the DNA and measuring the kinetics of its reassociation under standard conditions. The kinetics are expressed in terms of the convention C_0t, where C_0(mol/l.) is the initial concentration of the DNA and t(sec) is the time allowed for reassociation. It is an empirical observation that the complexity of the nucleotide sequences participating in the reaction is directly proportional to the value of C_0t at which half of the DNA has reassociated ($C_0t_{1/2}$) (Britten and Kohne, 1968).

This technique has been applied to show that in the endogenous RNA-directed DNA synthesis of detergent-disrupted RSV 85% of the ds-DNA product is transcribed from only 5% of the RSV genome (Varmus *et al.*, 1971). A similar result has been obtained with DNA synthesized by the purified enzyme using 70S RSV RNA as template (Taylor *et al.*, 1972, and also Table V and Figure 3).

TABLE III: Identification of the Covalent Link between a Deoxy- and a Ribonucleotide in the Product of the Purified Polymerase.[a]

RNA Template (2 μg/ml)	Exogenous Primer Concn (μg/ml)	% Transfer of α-^{32}P from dATP to			
		Cp	Ap	Gp	Up
RSV	0	<0.03	3.68	<0.02	<0.02
RSV	0.1	<0.5	3.69	<0.5	<0.5
RSV (denatured)	0.1	<0.6	<0.6	<0.6	<0.6

[a] DNA was synthesized with purified polymerase and 70S RNA as template in a standard reaction of 1 hr. It was then treated with sodium dodecyl sulfate and passed through a column of Sephadex G-50, treated with alkali (0.3 N NaOH, 18 hr, 37°), and subjected to high-voltage electrophoresis at pH 3.5, as previously described (Taylor *et al.*, 1972). The radioactive spots were located by autoradiography, cut out, and counted in a liquid scintillation counter. The data are expressed as the percentage of total radioactivity recovered from the electropherogram in each of the ribomononucleotides when [α-^{32}P]dATP is used as the labeled precursor. The remainder of the radioactivity was found at the origin as unhydrolyzed DNA. The results obtained in the presence of $(dT)_{12-18}$ have been normalized to correct for the stimulation of DNA synthesis by the exogenous primer (see Table I). Experiments using the other three possibilities of [α-^{32}P]-deoxynucleoside triphosphate failed to show significant transfer of label to a ribonucleotide. The percentage shown for transfer of ^{32}P from [α-^{32}P]dATP to Ap in reactions using RSV as template is the mean of four determinations and has a standard error of 0.39.

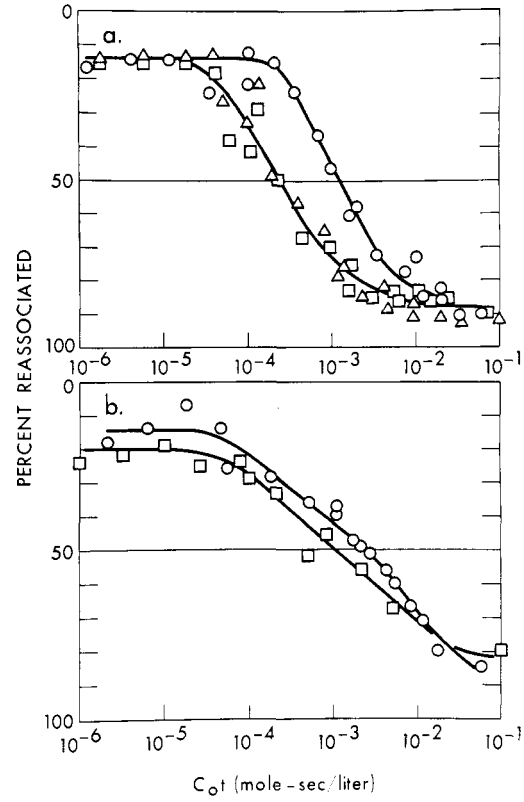

FIGURE 3: The extent of transcription into ds-DNA as measured by reassociation kinetics. ds-DNA was isolated from standard 18-hr reactions with the purified polymerase using [^{3}H]TTP as the labeled nucleotide precursor. Reassociation kinetics of the denatured DNAs in 0.4 M phosphate buffer were determined as previously described by Varmus *et al.* (1971). In part a, the template used was RSV RNA in either the absence (○) or presence of primer $(dT)_{12-18}$ (□), or in the presence of primer after initial denaturation of the template RSV RNA (△). In part b, the template was poliovirus RNA in either the absence (○) or presence of primer $(dT)_{12-18}$ (□). The template was used at 2 μg/ml and the primer at 0.1 μg/ml. The C_0t values have been corrected to standard conditions (Britten and Kohne, 1968).

The ds-DNA synthesized using poliovirus 35S RNA as template has approximately the same complexity as that obtained with RSV RNA (Figure 3b and Table V), although it should be noted that the range over which reassociation occurs is relatively wide, indicating that species with different rates of reassociation are present (Britten and Kohne, 1968).

In the presence of exogenous primer $(dT)_{12-18}$, the DNA products of an extended reaction also contain predominantly ds-stranded DNA (Table VI). This ds-DNA is less complex than ds-DNA synthesized under similar conditions in the absence of exogenous primer (Figure 3 and Table V).

Sequence Homology in DNA Products. Reassociation experiments were used to compare the sequence homology of ds-DNA synthesized by the purified enzyme with that synthesized by the endogenous reaction of detergent-disrupted virions. Two [^{3}H]DNA products of the purified enzyme were so tested: that synthesized using 70S RVS RNA and that using $(dT)_{12-18}$ plus denatured RSV RNA. They were each incubated in the absence or presence of a 400-fold excess of [^{32}P]DNA synthesized by the endogenous reaction of detergent-disrupted RSV. The conditions were such that the [^{32}P]DNA would reassociate but that the [^{3}H]DNA alone would not. The [^{32}P]-DNA was able to form duplexes with both species of [^{3}H]DNA (Table VII), as assayed by the single strand-specific deoxyribonuclease of *Aspergillus oryzae*. The above results indicate that the purified polymerase with or without exogenous primer is unable to transcribe into appreciable amounts of ds-DNA

TABLE IV: Identification of the Covalent Link between a Deoxy- and a Ribonucleotide in the Product of the Endogenous Reaction of Detergent-Disrupted Virions.[a]

[α-^{32}P]-Nucleoside Triphosphate	% Transfer of α-^{32}P to			
	Cp	Ap	Gp	Up
dATP	<0.07	9.93	<0.09	<0.07
dCTP	<0.03	<0.02	<0.02	<0.01
dGTP	<0.08	<0.03	<0.03	<0.02
dTTP	<0.09	<0.04	<0.03	<0.02

[a] DNA was synthesized in standard reactions (of 30 min) using the labeled nucleoside triphosphate as indicated above and purified RSV disrupted with NP40 (at 0.01% final). After the addition of sodium dodecyl sulfate (to 0.5%) the reaction mixtures were subjected to velocity sedimentation analysis on 15–30% sucrose gradients (as in Figure 2) at 40,000 rpm for 3 hr at 4° in a SW-41 rotor. In each case the 70S hybrid was isolated and precipitated with ethanol. Subsequent digestion with alkali and electrophoresis was as described in Table III.

TABLE V: Extent of Transcription of RNA Templates into ds-DNA.[a]

RNA Template (2 μg/ml)	Exogenous Primer (0.1 μg/ml)	Complexity of DNA Product (Nucleotide Pairs)
RSV	−	820 (3)
RSV	+	130 (0.5)
RSV (denatured)	+	130 (0.5)
Poliovirus	−	1480 (20)
Poliovirus	+	490 (7)

[a] The complexity values were deduced using the data of Figure 3 and appropriate standards as described previously (Taylor *et al.*, 1972). The figures in parentheses refer to the percentage of the RNA genome transcribed into ds-DNA, deduced using 2.5×10^6 and 1×10^7 for the molecular weights of poliovirus and RSV RNA, respectively.

any regions of the RSV genome other than those already accessible to the crude polymerase in the endogenous reaction of detergent-disrupted virions. This conclusion applies even when the purified polymerase is directed by denatured RSV RNA in the presence of the exogenous primer $(dT)_{12-18}$.

RNA-Directed DNA Synthesis with E. coli Polymerase I. The response of this bacterial polymerase to naturally occurring RNA molecules is less than 1% of that obtained with calf thymus DNA (Table VIII). Such a response is very low relative to that obtained with the viral polymerase where the response to RSV RNA is about 40% of that with DNA (Faras *et al.*, 1972).

However, in the presence of $(dT)_{12-18}$, the response of the bacterial polymerase to certain naturally occurring RNA templates is high, even exceeding the response obtained with calf thymus DNA (Table VIII). This stimulation is *only* observed when the labeled precursor in the reaction mixture is either dATP or dTTP (Table VIII and X). RSV and poliovirus RNA, but not rRNA (data not shown) or phage R17 RNA, exhibit this effect. The data are consistent with the interpretation that a poly(A) region is needed in the template. Nearest-neighbor analysis indicates that the DNA product of a 2-hr reaction using RSV RNA in the presence of $(dT)_{12-18}$ is composed of poly(dA) and poly(dT) (Table IX). Analysis on hydroxylapatite after treatment with pancreatic ribonuclease (at 100 μg/ml for 1 hr at 37°) indicates that the product is predominantly double stranded (Table X). Centrifugation to equilibrium in Cs_2SO_4 (data not shown) indicates a density consistent with DNA, thereby eliminating the possibility that the double-stranded behavior on hydroxylapatite may actually be due to poly(A)·poly(dT). The kinetics of reassociation of the ds-DNA yield a $C_0t_{1/2}$ of 8×10^{-6} (mol sec)/l. (Figure 4), suggesting a very low complexity, comparable to that for poly(A)·poly(U) ($2-3 \times 10^{-6}$ (mol sec]/l.) (Britten and Kohne, 1968). The present data are therefore consistent with a complexity of at most only a few base pairs.

Evidence for the mechanism of synthesis of the poly(dA)·poly(dT) was obtained as follows. When dATP was omitted from the reaction mixture, the [^{3}H]TTP incorporated into DNA product, after treatment with ribonuclease, eluted as ss-DNA from hydroxylapatite analysis (Table X). When TTP was omitted from the reaction mixture, the incorporation of [^{3}H]dATP was reduced to background levels (Table X). These data suggest that in the presence of $(dT)_{12-18}$, the bacterial enzyme is able to synthesize DNA from those RNA templates that possess poly(A) sequences. This DNA product is poly-

TABLE VI: Synthesis of ds-DNA.[a]

RNA Template	Percentage of ds-DNA	
	$-(dT)_{12-18}$	$+(dT)_{12-18}$
RSV	71 (58)	72 (61)
RSV (denatured)		88 (67)
Poliovirus	68 (66)	61 (59)

[a] DNA was synthesized in a standard reaction of 18 hr using [^{3}H]TTP as the labeled precursor. Following treatment of the extracted DNA product with ribonuclease to disrupt DNA–RNA hybrids (Fanshier *et al.*, 1971), the secondary structure was assayed by fractionation on hydroxylapatite and by the use of the single strand-specific nuclease from *Aspergillus oryzae* (Leong *et al.*, 1972), the latter results being shown in parentheses. The results were standardized with native and denatured DNA from phage λ.

TABLE VII: Homologous Sequences among ds-DNA Products.[a]

Expt	ds-DNAs in Reass Mix	% Reassociation of [^{3}H]DNA	% Reassociation of [^{32}P]DNA
1	[^{3}H]DNA (synthesized by purified polymerase using 70S RSV RNA template)	18 (15)	
	As above + [^{32}P]DNA (synthesized by endogenous reaction of holoenzyme)	80 (82)	77 (75)
2	[^{3}H]DNA (synthesized by purified polymerase using denatured RSV RNA plus $(dT)_{12-18}$	13	
	As above + [^{32}P]DNA (synthesized by endogenous reaction of holoenzyme)	76	83

[a] Labeled DNA was synthesized in standard reactions of 18-hr duration with TTP as the labeled precursor and with conditions of enzyme, template and primer as described in the table. ds-DNA was isolated using hydroxylapatite and subsequently passed through a column of Sephadex G-50. The [^{3}H]DNA in the presence or absence of a 400-fold excess of [^{32}P]DNA was denatured and then allowed to reassociate in 0.6M NaCl to a [^{32}P]DNA C_0t of 0.16 and 0.012 (mol sec) per l. for expt 1 and 2, respectively. The extent of reassociation of the ds-DNA was then assessed by use of a single strand-specific nuclease from *A. oryzae* (Leong *et al.*, 1972). For expt 1, reassociation was also measured at the higher [^{32}P]DNA C_0t of 0.4 (mol sec)/l. and the results are shown in parentheses. The reassociation of [^{3}H]DNA in the presence of [^{32}P]DNA had reached a plateau at the lower C_0t value.

TABLE VIII: Effect of $(dT)_{12-18}$ on the Response of *E. coli* Polymerase I to Various RNAs.[a]

Template (2 μg/ml)	Exogenous Primer Concn (μg/ml)	Incorporation When Labeled Nucleoside Triphosphate Is			
		dCTP	dATP	dGTP	dTTP
Calf thymus DNA	0	1.00	1.00	1.00	1.00
RSV RNA	0	nd	nd	0.003	0.003
RSV RNA	0.1	nd	nd	0.003	0.110
RSV RNA	2	nd	nd	0.003	1.20
Poliovirus RNA	0	0.010	0.013	0.003	0.003
Poliovirus RNA	0.1	nd	nd	0.003	1.55
Poliovirus RNA	2	0.067	3.20	0.003	7.98
R17 RNA	0	nd	nd	0.003	0.003
R17 RNA	0.1	nd	nd	0.003	0.003
R17 RNA	2	nd	nd	0.003	0.003

[a] Standard reaction mixtures (0.05 ml), modified in that RSV polymerase was replaced by 4 units/ml (according to definition in Materials and Methods section) of *E. coli* polymerase I fraction VII (a gift from Dr. J. Huberman), were incubated for 2 hr at 37°. The incorporation of a particular labeled nucleotide (at 8000 ^{3}H cpm/pmol) under different conditions of template and primer are expressed relative to the incorporation obtained when the template is calf thymus DNA (2 μg/ml). These incorporations were 9.3, 2.2, 5.6, and 11.7 pmol for reactions mixtures using ^{3}H-labeled dCTP, dATP, dGTP, and dTTP, respectively. Those assays not done in this experiment are shown as nd.

(dT) which is subsequently transcribed to yield the double-stranded molecule, poly(dA)·poly(dT).

Discussion

Transcription of RNA Templates. The DNA product arising from the transcription of RSV RNA by the purified polymerase of RSV is small (*ca.* 4 S) relative to the 70S template. Moreover, the fraction of the product that is double stranded consists primarily of species representing only 3% of the genome (Table VI and Taylor *et al.*, 1972). The present experiments also show that the polymerase will transcribe the smaller 35S RNA to poliovirus, albeit with a lower efficiency (Table I). The DNA products are small and the ds-DNA, although it represents synthesis predominantly from as much as 20% of the template, is not of significantly greater complexity than the product of RSV RNA transcription (Figure 3 and Table V).

What causes these limitations of size and complexity? Deoxyribonucleases have been reported as present in preparations of purified tumor virus particles (Mizutani *et al.*, 1970, 1971; Quintrell *et al.*, 1970; Mölling *et al.*, 1971; Hurwitz and Leis, 1972) but neither we nor others (Leis and Hurwitz, 1972) have been able to detect such activity in purified enzyme. Ribonuclease is active in detergent-disrupted virions (Quintrell *et al.*, 1970), but we have previously demonstrated that in reactions using purified polymerase the limited size of the

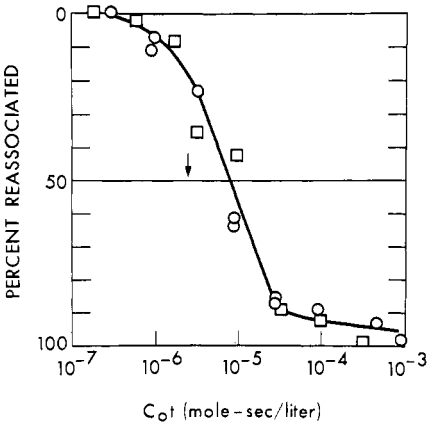

FIGURE 4: Reassociation kinetics of ds-DNA synthesized by the *E. coli* polymerase I. ds-DNA was synthesized and isolated as described in Figure 3, using either RSV (○) or poliovirus (□) RNA as template, in the presence of $(dT)_{12-18}$. Reassociation kinetics in 0.4 M phosphate buffer were measured as described previously (Taylor *et al.*, 1972). The arrow shows the $C_0t_{1/2}$ of poly(A)·poly(U), computed by Britten and Kohne (1968) from data of Ross and Sturtevant (1962).

TABLE IX: Nearest-Neighbor Analysis of *E. coli* Polymerase I Product.[a]

^{32}P-Labeled Precursor	Percentage of Label Transferred to			
	dCp	dAp	dGp	dTp
dATP	0.3	98.1	1.4	0.2
dTTP	0.1	2.4	0.2	97.3

[a] *E. coli* polymerase I was used at 4 units/ml in standard reactions with α-^{32}P-labeled nucleoside triphosphate as indicated above. RSV RNA template was used at 2 μg/ml in the presence of 0.1 μg/ml of $(dT)_{12-18}$. After 2 hr at 37° the reactions were terminated and the DNA products were extracted and analyzed for nearest-neighbor analysis as described for Table II.

TABLE X: Response of *E. coli* Polymerase I to RNA.[a]

Reaction Mixture		Enzyme Response to Template RNA	
^{3}H-Labeled Precursor	Modifications	RSV	Poliovirus
dATP	None	1084 (94)	3739 (101)
dATP	Minus dTTP	0	0
dTTP	None	2416 (80)	7719 (102)
dTTP	Minus dATP	1618 (18)	1307 (19)

[a] *E. coli* polymerase I was used at 4 units/ml in standard reaction mixtures with ^{3}H-labeled precursor and modifications (if any) as indicated above. RNA templates were used at 2 μg/ml in the presence of $(dT)_{12-18}$ at 0.1 μg/ml. After 3 hr at 37° the reactions were terminated and aliquots were removed for the assay of incorporation of ^{3}H into acid-insoluble material. The remainder of the product was prepared as described in Table II, treated with ribonuclease in low salt, and fractionated on hydroxylapatite. The percentage of the product that behaved as ds-DNA is shown in parentheses.

product is probably not due to degradation of the template (Faras *et al.*, 1972).

One interpretation is that in RNA-directed synthesis (at least when tested under the present conditions), after initiation at an RNA primer, polymerization can only proceed a limited distance along the template before there is inhibition due to secondary structure of the template. Synthesis is then aborted yielding only short-term products of approximately the same size.

What are the consequences of this interpretation? The modal size of the DNA products is about 80 nucleotides and yet the complexity of the ds-DNA is consistent with transcription from 820 to 1480 nucleotides of RNA. For RSV RNA, where initiation appears to occur on a low molecule weight RNA primer (Faras *et al.*, 1972),[2] there must be at least 10–20 potential primer sites on each 70S RNA molecule. Of course, it is not necessary to imply that all possible primer sites are available and/or utilized on *each* 70S RNA molecule. For poliovirus RNA the nature of the "primer" has not yet been established. This primer is intrinsically different from that of RSV in that it cannot be permanently removed by denaturation (unpublished observation). Presumably the poliovirus RNA renatures and initiation is achieved by the formation of a structure which allows the 3′-OH terminus of the molecule to act as a primer. Leis and Hurwitz (1972) have presented evidence that this mechanism occurs when phage f2 RNA is used as a template. Since the above model predicts 10–20 different initiation sites, it can be concluded that the 3′-OH terminus of poliovirus RNA must be able to form a structure suitable for initiation at 10–20 different sites along the RNA.

Effect of Exogenous Primer on Transcription of RNA Templates. When the exogenous primer $(dT)_{12-18}$ is added to the reaction mixture, the response of the polymerase to certain RNA templates is increased (Figure 1 and Table I). In fact, the data are consistent with the interpretation that $(dT)_{12-18}$ will only give a stimulation if the RNA template possesses poly(A) sequences. Poly(A) is present in RSV RNA (Lai and Duesberg, 1972; Green and Cartas, 1972; Gillespie *et al.*, 1972) and poliovirus RNA (Armstrong *et al.*, 1972). It is absent from phage R17 RNA (Gillespie *et al.*, 1972) and rRNA (Sheldon *et al.*, 1972). The bulk of the DNA synthesized from poliovirus and RSV RNA in the presence of $(dT)_{12-18}$ is only slightly larger (Figure 2) and is certainly not more complex than that synthesized in its absence (Figure 3 and Table V). In addition, while the $(dT)_{12-18}$ provides more sites of initiation, it does not interfere with initiation on the intrinsic primer(s) of RSV RNA (Table III). In fact, the ds-DNA synthesized includes few or no nucleotide sequences other than those which can be synthesized by the endogenous reaction of detergent-dirupted virions (Table VII).

Under the present conditions of DNA synthesis by the polymerase, with different RNA templates, and in either the presence or absence of $(dT)_{12-18}$, there seems to be an upper limit to the size of the DNA product. Other laboratories have used the exogenous primer $(dT)_{12-18}$, to induce the oncornavirus polymerase to transcribe globin mRNA (Verma *et al.*, 1972b; Ross *et al.*, 1972; Kacian *et al.*, 1972), yielding a DNA product that is primarily 6–9 S in size, very close to the total size of the 10S mRNA template. To date, this is the largest RNA template which has been transcribed into DNA chains coextensive in length with the template. We presume that with such a relatively small template the polymerase is less restricted by the secondary structure of the template.

[2] Submitted for publication.

Nature of Covalent Linkage between RNA Primer and DNA Product. For both the purified reaction and the endogenous reaction of detergent-disrupted RSV, we find that the 3′-hydroxyl terminus of the primer RNA is adenosine which during DNA synthesis becomes covalently linked to deoxyadenosine. Using avian myeloblastosis virus, Verma *et al.* (1972a) have obtained the same result for both the endogenous and reconstructed reaction. Flügel and Wells (1972) studying only the endogenous reaction of ether-disrupted avian myeloblastosis virus observe the same transfer of ^{32}P from [α-^{32}P]-dATP to Ap, but by contrast obtain a predominant transfer from [α-^{32}P]dCTP to Up. There is currently no explanation for this disagreement.

Transcription of RNA by E. coli DNA Polymerase I. The DNA-dependent DNA polymerase I of *E. coli* does not transcribe naturally occurring RNA, a finding in agreement with recent work by Goodman and Spiegelman (1971), Robert *et al.* (1972), and Smoler (referred to in Temin and Baltimore, 1973). In the presence of both $(dT)_{12-18}$ and RSV or poliovirus RNA (both known to possess sequences of poly(A)), we observe synthesis only of poly(dT) and a somewhat lesser amount of poly(dA), copied from the poly(dT) product. Cavalieri and Carroll (1970, 1971) using similar but not identical reaction conditions have claimed that *E. coli* DNA polymerase (step 4) will transcribe 5S and rRNA. Their DNA product is composed almost entirely of dA and dT, and is thus similar to the product described in the present communication, although synthesis apparently occurred without added primer.

Implications of the Limited Transcription of RNA Templates. The endogenous reaction of RNA tumor virus particles disrupted with nonionic detergent makes possible the synthesis of DNA with a specific activity as high as 2×10^7 cpm of $^3H/\mu g$. Small amounts of this DNA (0.0001 μg), used in conjunction with hydroxylapatite analysis or a single strand-specific nuclease, provides an extremely sensitive reagent for nucleic acid hybridization. Such ss-DNA product has been used to detect RNA sequences in cells (Garapin *et al.*, 1971; Green, 1972; Coffin and Temin, 1972; Leong *et al.*, 1972). The double-stranded product has been used to measure complementary DNA in cells (Gelb *et al.*, 1971; Varmus *et al.*, 1972). The utility of both the single- and double-stranded probes is limited by the fact that neither form of DNA provides a uniform representation of all nucleotide sequences in the virual genome (Gelb *et al.*, 1971; Varmus *et al.*, 1971; Bishop *et al.*, 1972).

It might have been reasonable to suggest that this limitation of the probe could be solved by: (1) use of purified polymerase, (2) denaturation of the template RNA, (3) synthesis in the presence of exogenous primers, *e.g.*, $(dT)_{12-18}$. The present communication shows that in the transcription of RSV *and* poliovirus RNA, none of these variables are able to make the ds-DNA product more representative of the RNA from which it is transcribed.

Acknowledgments

We thank L. Levintow for support and editorial assistance and N. Quintrell, K. Smith, L. Fanshier, B. Evans, and J. Jackson for expert technical assistance.

References

Armstrong, J. A., Edmonds, M., Nakazato, H., Phillips, B. A., and Vaughan, M. H. (1972), *Science 176*, 526.

Bishop, J. M., Faras, A. J., Garapin, A. C., Hansen, C., Jackson, N., Levinson, W., Taylor, J. M., and Varmus, H. E. (1972), The Molecular Basis of Neoplasia, Houston, Texas, Houston Press (in press).

Britten, R. J., and Kohne, D. E. (1968), *Science 161*, 529.

Cavalieri, L. F., and Carroll, E. (1970), *Biochem. Biophys. Res. Commun. 41*, 1055.

Cavalieri, L. F., and Carroll, E. (1971), *Nature (London) 232*, 254.

Coffin, J. M., and Temin, H. M. (1972), *J. Virol. 9*, 766.

Duesberg, P., Helm, K. v. d., and Canaani, E. (1971a), *Proc. Nat. Acad. Sci. U. S. 68*, 747.

Duesberg, P., Helm, K. v. d., and Canaani, E. (1971b), *Proc. Nat. Acad. Sci. U. S. 68*, 2505.

Fanshier, L., Garapin, A. C., McDonnell, J. P., Faras, A., Levinson, W., and Bishop, J. M. (1971), *J. Virol. 7*, 77.

Faras, A. J., Taylor, J. M., McDonnell, J. P., Levinson, W. E., and Bishop, J. M. (1972), *Biochemistry 11*, 2334.

Flügel, R. M., and Wells, R. D. (1972), *Virology 48*, 394.

Garapin, A. C., Leong, J., Fanshier, L., Levinson, W. E., and Bishop, J. M. (1971), *Biochem. Biophys. Res. Commun. 42*, 919.

Gelb, L. D., Aaronson, S. A., and Martin, M. A. (1971), *Science 172*, 1353.

Gillespie, D., Marshall, S., and Gallo, R. C. (1972), *Nature (London), New Biol. 236*, 227.

Goodman, N. C., and Spiegelman, S. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2203.

Green, M. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1036.

Green, M., and Cartaes, M. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 791.

Hurwitz, J., and Leis, J. P. (1972), *J. Virol. 9*, 116.

Kacian, D. L., Spiegelman, S., Bank, A., Terada, M., Metafora, S., Dow, L., and Marks, P. A. (1972), *Nature (London), New Biol. 235*, 167.

Kacian, D. L., Watson, K. F., Burny, A., and Spiegelman, S. (1971), *Biochim. Biophys. Acta 246*, 365.

Lai, M., and Duesberg, P. H. (1972), *Nature (London) 235*, 383.

Leis, J. P., and Hurwitz, J. (1972), *J. Virol. 9*, 130.

Leong, J., Garapin, A., Jackson, N., Fanshier, L., Levinson, W., and Bishop, J. M. (1972), *Virology 9*, 891.

Mizutani, S., Boettiger, D., and Temin, H. M. (1970), *Nature (London) 228*, 424.

Mizutani, S., Temin, H. M., Kodama, M., and Wells, R. D. (1971), *Nature (London), New Biol. 230*, 232.

Mölling, K., Bolognesi, D. P., and Bauer, H. (1971), *Virology 45*, 298.

Quintrell, N., Fanshier, L., Evans, B., Levinson, W., and Bishop, J. M. (1971), *J. Virol. 8*, 17.

Robert, M. S., Smith, R. G., Gallo, R. C., Sarin, P. S., and Abrell, J. W. (1972), *Science 176*, 798.

Ross, J., Aviv, H., Scholnick, E., and Leder, P. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 264.

Ross, P. D., and Sturtevant, J. M. (1962), *J. Amer. Chem. Soc. 84*, 4503.

Sheldon, R., Jurale, C., and Kates, J. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 417.

Smoler, D., Molineaux, I., and Baltimore, D. (1972), *J. Biol. Chem. 246*, 7697.

Taylor, J. M., Faras, A. J., Varmus, H. E., Levinson, W. E., and Bishop, J. M. (1972), *Biochemistry 11*, 2343.

Temin, H. M., and Baltimore, D. (1973), *Advan. Virus Res.* (in press).

Varmus, H. E., Levinson, W. E., and Bishop, J. M. (1971), *Nature (London), New Biol. 233*, 19.

Varmus, H. E., Weiss, R. A., Friis, R. R., Levinson, W. E., and Bishop, J. M. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 20.

Verma, I. M., Meuth, N. L., and Baltimore, D. (1972a), *J. Virol. 10*, 622.

Verma, I. M., Meuth, N. L., Bromfield, E., Manly, K. F., and Baltimore, D. (1971), *Nature (London), New Biol. 233*, 131.

Verma, I. M., Temple, G. E., Fan, H., and Baltimore, D. (1972b), *Nature (London), New Biol. 235*, 163.