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Enzymatic Synthesis of DNA Complementary to Purified 14S Messenger RNA of Immunoglobulin Light Chain

(RNA-dependent DNA polymerase/hybridization/MOPC 41/mouse)

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ABSTRACT **The 14S messenger RNA, which contains poly(adenylic acid), of MOPC 41 (mouse plasmocytoma) immunoglobulin light chain, purified to a single peak as shown by polyacrylamide gel electrophoresis, was used to synthesize complementary DNA with the RNA-dependent DNA polymerase of avian myeloblastosis virus. DNA synthesis is entirely dependent on added RNA template and oligo(dT) primer. Both the size and the concentration of the primer affect the reaction. The product behaves similarly to DNA during centrifugation in cesium sulfate density gradients. It is shown by hybridization that the DNA made is complementary to the purified template, light-chain mRNA. The high specific activity of the complementary DNA should make it suitable for gene-dosage experiments. According to alkaline sucrose gradient analyses, some complete complementary DNA transcripts of the 14S mRNA seem to be made. Oligo(dG) can also function as a primer for DNA synthesis, possibly by annealing to an internal cluster of cytidines in the mRNA, that correspond to the bases coding for amino-acids 119 and 120 of the MOPC 41 light chain.**

Two major theories have been proposed to explain the generation of antibody diversity: the germ line theory and theories that involve somatic mutations and recombination (for a review, see ref. 1). Both theories postulate the formation of a large number of immunoglobulin genes with only minor differences in nucleotide sequence, such sequence differences corresponding to the differences in the amino-acid sequence of different antibody molecules. These differences are located in the variable part of the polypeptide chain. It is postulated that the light chain is coded for by two different genes, the constant and the variable genes, and that antibody diversity results from an extensive duplication of variable genes occurring either during evolution (germ line theory) or during differentiation (somatic mutation theory). Studies of the amino-acid sequences of immunoglobulins have not permitted a decision between these major theories. However, a direct determination of the number of genes for immunoglobulins, in particular of the genes for the variable part in the DNA of germ line and somatic tissues, could help decide between the alternative theories.

Hybridization of labeled mRNA with a large excess of cellular DNA has not been successful because high enough specific activities of the mRNA might not be achieved with *in vivo* labeling. RNA or DNA synthesized *in vitro*, however, is labeled with a high enough specific activity to allow hybridization with a large excess of unlabeled DNA. RNA-dependent DNA polymerase (2, 3) from avian myeloblastosis virus has been used to synthesize labeled complementary DNA from hemoglobin mRNA (4–6) and from vaccinia virus mRNA (7) with oligo(dT) as primer in the reaction.

We have recently shown that an RNA fraction obtained from polysomes released from microsomes of a mouse myeloma tumor, and containing long sequences of poly(A), can serve as a template for complementary (c)DNA synthesis (8). However, only cDNA synthesized from a purified mRNA could give meaningful hybridization data for gene-dosage experiments. From the RNA fraction that contains poly(A) mentioned above, a 14S mRNA has been purified to a single peak by sucrose gradient centrifugation and polyacrylamide gel electrophoresis (9). The molecular weight has been estimated to be 380,000, which corresponds to about 1100 nucleotides. This RNA directs the synthesis of light chain *in vitro* (reticulocyte lysate) and *in vivo* (*Xenopus laevis* ooctyes). The immediate product of the light chain 14S mRNA in the reticulocyte lysate migrates slightly slower on sodium dodecyl sulfate (SDS) gels and appears to be a precursor about 20 amino acids longer than the light chain. It is this purified light chain mRNA that is used in the following experiments for synthesis of cDNA specific for light chain.

MATERIAL AND METHODS

Purification of 14S mRNA of Immunoglobulin Light Chain. The preparation of membrane-bound polysomes from mouse myeloma tumor MOPC 41 (gift of Dr. M. Potter), extraction of RNA, and isolation of an RNA fraction containing poly-(A) by poly(dT) chromatography has been described (8). The purification from this RNA fraction, containing poly(A), of 14S mRNA of MOPC 41 light chain by successive sucrose gradient centrifugations is also described (9). The symmetrical 14S RNA peak was precipitated with ethanol and stored in H_2O at $-30°$.

Conditions for Enzymatic Synthesis of DNA. The purification of RNA-dependent DNA polymerase of avian myeloblastosis virus has been described (8). Unless otherwise indicated in legends, a 20-μl incubation mixture contains: 54 μg/ml of purified enzyme, 1 mM unlabeled dTTP, dCTP, and dATP, 10 mM KCl, 50 mM Tris·HCl (pH 7.5), 1.5 mM 2-mercaptoethanol, 25 μg/ml of actinomycin D to prevent the formation of double-stranded DNA (4), and 5 μM [^{3}H]dGTP (8600 cpm/pmol, Amersham). 14S mRNA and oligodeoxynucleotide primer were added as indicated. When indicated, [^{3}H]dCTP (6700 cpm/pmol) or [^{3}H]dTTP (8600 cpm/pmol), both from Amersham, was used. Samples were incubated for 20 min at 37°, precipitated with Cl_3CCOOH, and counted (8).

Centrifugation of DNA in Alkaline Sucrose Gradients. After incubation for DNA synthesis, the samples were

Abbreviation: cDNA, complementary DNA; SDS, sodium dodecyl sulfate.

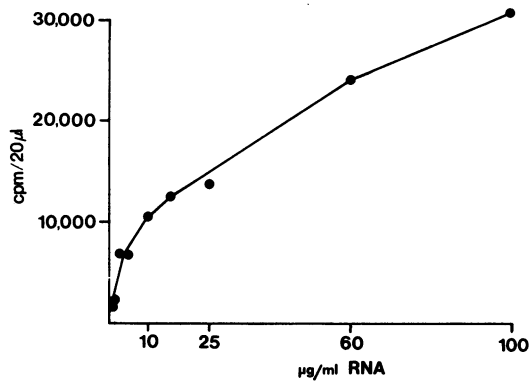

FIG. 1. Dependence of [^{3}H]dGTP incorporation on the concentration of 14S mRNA template. Incorporation was measured as described in *Methods* with increasing concentrations of 14S mRNA. The oligo(dT) primer used was that obtained by digestion of poly(dT) with 200 μg/ml of DNase as described in Fig. 2. In order to keep the ratio of primer to RNA constant, the concentration of oligo(dT) added to each sample was equal to 0.1 of the mRNA concentration.

brought to a final concentration of 1% SDS and 0.2 N NaOH, and layered on a 5–20% linear sucrose gradient in 0.8 M NaCl–0.2 N NaOH–10 mM EDTA. The gradients were centrifuged for 14 hr at 45,000 rpm in a Spinco SW50.1 rotor at 4°. Fractions were collected, precipitated with Cl_3CCOOH, and counted.

Hybridization Experiments. After incubation for DNA synthesis, the samples were treated with 0.4 N NaOH for 20 min at 80° and centrifuged through an alkaline sucrose gradient (see above). The DNA peak was collected, neutralized, and precipitated with alcohol in the presence of 10 μg of T4 phage DNA. Hybridization to different RNAs was performed in 10-μl total volume, containing 0.3 M NaCl, 40 mM Tris·HCl (pH 7.5), and 0.1% SDS. The mixture was sealed in a 50-μl tube ("Microcap") for 2 hr at 65°. The samples were then added to 200 μl of 30 mM sodium acetate (pH 4.6)–1 mM $ZnSO_4$–5% glycerol–50 mM NaCl containing 4 units of nuclease from *Aspergillus oryzae* specific for single strands (generous gift of V. Vogt) (1 unit = 10 μg of DNA digested in 10 min at 45°) and 5 μg of denatured, sonicated salmon-sperm DNA. After incubation at 45° for 30 min, the sample was precipitated with Cl_3CCOOH and counted.

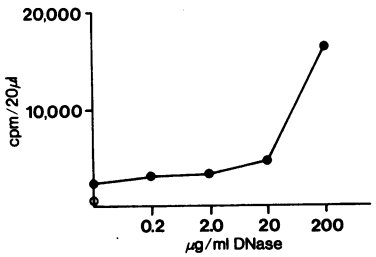

FIG. 2. Dependence of the incorporation of [^{3}H]dGTP on the size of the oligo(dT) primer. Poly(dT) (Collaborative Research) (60 μg/ml) was digested with pancreatic DNase (electrophoretically pure, Worthington) at different concentrations for 30 min at 37° to obtain primers of different lengths. The primers were treated with diethylpyrocarbonate (Bacovin, Bayer) for 5 min at room temperature (24°), followed by boiling for 10 min to destroy the nuclease. Each assay contained 10 μg/ml of 14S light chain mRNA and 3 μg/ml of oligo(dT) of each size. Assay conditions were as described in *Methods*. ●——●, poly(dT); O, background incorporation without primer.

RESULTS

Properties of the RNA-dependent DNA synthesis

The synthesis of DNA is completely dependent upon addition of RNA (Fig. 1) and of primer (Figs. 2 and 3). The incorporation depends on the size of the primer used in the reaction; the smallest oligo(dT) used is the most efficient primer (Fig. 2). The incorporation also depends on the concentration of primer used; a large excess of primer has a slight inhibitory effect (Fig. 3).

The mRNA-dependent incorporation of [^{3}H]dGTP no longer increases after 20–30 min (Fig. 4). In order to determine if this result was due to inactivation of the enzyme or of the template, we added fresh enzyme 15 min after the start of the reaction. This addition resulted in a second burst of incorporation (Fig. 4). At the low concentration of enzyme used in the reaction, and at 0°, the half-life of the enzyme was 30 min. The addition of 1 mg of bovine-serum albumin per ml doubled the incorporation (data not shown), probably by stabilizing the enzyme.

Characterization of the DNA product

The DNA synthesized was analyzed on cesium sulfate equilibrium density gradients, and data analogous to those obtained previously with total RNA containing poly(A) as template were found (see ref. 8). A small amount of radioactivity of the native product bands at the position of an RNA marker, while most of the counts band at the position of DNA. After heat denaturation, all of the radioactivity bands at the position of DNA. This behavior is expected for a DNA product that is made on an RNA template. When analyzed on alkaline sucrose gradients (Fig. 5), the product of the reaction with oligo(dT) is about 5 S, with some molecules sedimenting in the position of 10 S. These values are only approximate because they were obtained by comparison with bovine-serum albumin rather than with DNA markers, which are not readily available in that size range.

The following experiments were performed to investigate the possibility that the poly(A) region of the mRNA might be transcribed preferentially, or even exclusively. DNA synthesis

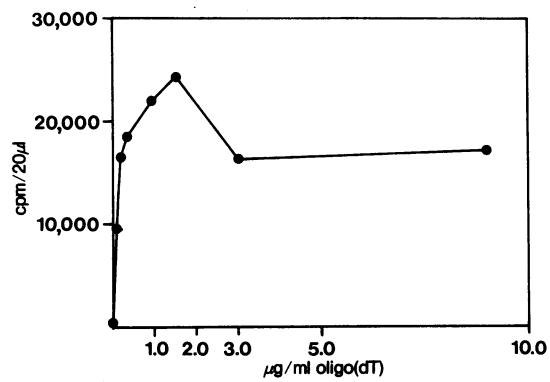

FIG. 3. Dependence of the incorporation of [^{3}H]dGTP on the concentration of oligo(dT) primer. The oligo(dT) primer obtained by digestion with 200 μg/ml of DNase (see Fig. 2) was used at increasing concentrations. The incorporation was measured as described in *Methods;* each assay contained 10 μg/ml of 14S mRNA.

TABLE 1. *Hybridization of DNA made with 14S light chain mRNA as template with RNA of different origins*

Experiment	cpm DNA input	cpm Hybridized	% Hybridized
cDNA alone	700	17	2.4
cDNA + 200 μg/ml 10S mRNA of hemoglobin	700	20	2.9
cDNA + 200 μg/ml of *E. coli* rRNA	700	20	2.9
cDNA + 20 μg/ml of 14S mRNA of light chain mRNA	700	497	71.1
cDNA + 200 μg/ml of 14S mRNA of light chain mRNA	700	617	88.1

Incubation for DNA synthesis and hybridization experiments were as described in *Methods*. Background of 20 cpm was subtracted. Rabbit reticulocyte polysomes were prepared (10), from which 10S hemoglobin mRNA was purified as described for 14S L-chain mRNA (see *Methods*).

was measured independently with different labeled deoxynucleoside triphosphates, and the relative incorporation of dTMP, dCMP, or dGMP was determined (Fig. 6). With the smallest oligo(dT) as primer, dTMP is incorporated more efficiently than dCMP or dGMP. It can be estimated from Fig. 6 that about 30% of the total incorporation results from transcription of the poly(A) portion of the 14S mRNA. When a large oligo(dT) primer is used, however, there is no preferential incorporation of dTMP (Fig. 6).

The DNA synthesized has been hybridized with RNA from different sources (Table 1) to study the specificity of the product made. From the low value found for self-annealing of the DNA (2.4%), we can conclude that only single-stranded DNA has been synthesized, as expected in the presence of actinomycin D (4). No hybridization could be observed with a large excess of either *Escherichia coli* ribosomal RNA or hemoglobin 10S mRNA from rabbit reticulocytes. Nearly complete annealing occurs, however, when the DNA product is hybridized back to the 14S light chain mRNA template.

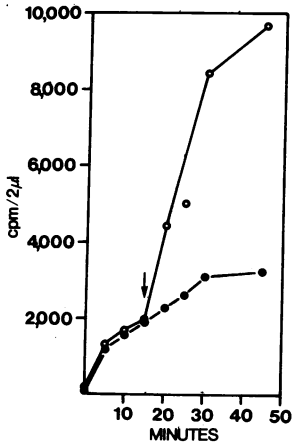

FIG. 4. Effect of addition of fresh enzyme to a reaction mixture 15 min after the beginning of [^{3}H]dGTP incorporation. Conditions were as described in *Methods*, with the 14S mRNA and oligo(dT) primer as in Fig. 1. Control (●——●). After 15 min of incubation, fresh enzyme (equal to the initial amount) was added to the reaction, and synthesis was continued for another 30 min (○——○). Aliquots of 2 μl were precipitated with Cl_3CCOOH and counted.

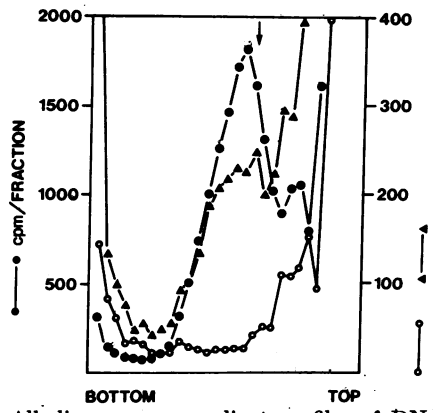

FIG. 5. Alkaline sucrose gradient profiles of DNA products made with 50 μg/ml of 14S L-chain mRNA and different primers (6 μg/ml). The DNA was synthesized under standard conditions (see *Methods*), either with oligo(dT) primer (as in Fig. 1) and 10 μM [^{3}H]dGTP (●——●), with $(dG)_6$ primer (Collaborative Research) and 10 μM [^{3}H]dGTP (▲——▲), or with $(dC)_6$ primer (Collaborative Research) and 13.5 μM [^{3}H]dCTP (○——○). With $(dA)_6$ primer, the results were the same as those obtained with $(dC)_6$. After incubation, samples were prepared and analyzed on alkaline sucrose gradients as described in *Methods*. Bovine-serum albumin (4.6 S) was run on a parallel neutral sucrose gradient, and its position, as determined by absorbance at 280 nm, is indicated by the *arrow*.

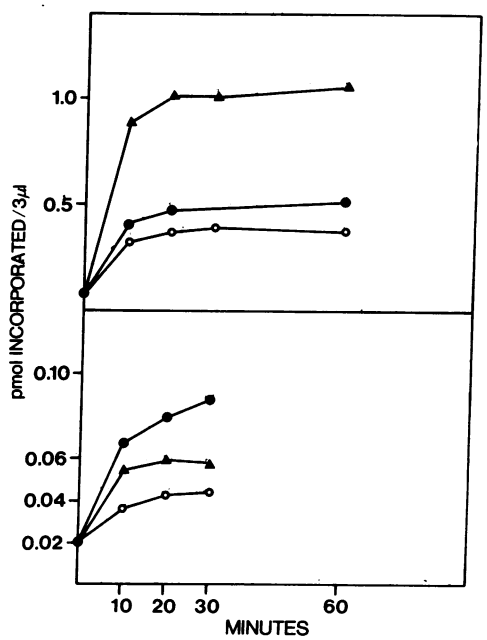

FIG. 6. Relative incorporation of labeled dTTP, dGTP, and dCTP in the presence of the smallest oligo(dT) primer (*top*) (200 μg/ml DNase, see Fig. 2), or of the largest oligo(dT) primer (*bottom*) (0.2 μg/ml DNase, see Fig. 2). The 14S mRNA (20 μg/ml) was used with 2 μg/ml of primer and 10 μg/ml of enzyme. Assay conditions were otherwise as indicated in *Methods*. The results are expressed in pmol incorporated per 3-μl aliquot. ▲——▲, [^{3}H]dTTP; ●——●, [^{3}H]dGTP; ○——○, [^{3}H]dCTP.

DNA synthesis with oligo(dG) as primer

In the above experiments, DNA synthesis was obtained with oligo(dT) as primer, which presumably anneals to the poly(A) sequence on the 3′ end of the mRNA template. We have investigated the possibility that segments other than the poly(A) sequence could bind oligodeoxynucleotide primers and provide initiation points for DNA synthesis within the mRNA template molecule. Therefore, DNA synthesis was measured with oligo(dC), oligo(dA), or oligo(dG) as primers. We found that the use of oligo(dG) results in a significant synthesis of DNA, but that no DNA synthesis is observed with oligo(dC) or oligo(dA) (Fig. 5). Although the overall synthesis is lower with oligo(dG) than with oligo(dT), the size of the DNA product synthesized is about the same in both cases (Fig. 5).

DISCUSSION

The availability of purified immunoglobulin light chain 14S mRNA has made possible the synthesis of complementary DNA that is specific for the light chain. Hybridization data showed that the DNA synthesized is indeed complementary to the mRNA template used. The high specific activity of this cDNA should make it suitable for hydridization studies with a large excess of DNA.

Most of the DNA made is about 5S, with some molecules as large as 10S. DNA coding for the 700 nucleotides of 14S mRNA that are translated into light chain would have a sedimentation coefficient of about 8S, according to Studier (11). DNA complementary to the entire 14S RNA would be about 9S.

When fresh enzyme is added to the incubation mixture after 15 min of incubation, a new burst of [^{3}H]dGTP incorporation is observed. This result indicated that some mRNA template is still available for transcription and that the early plateau in DNA synthesis is more likely to be due to inactivation of enzyme than to degradation of the mRNA by RNase H (12) contained in the polymerase preparation or by other RNase activities.

The studies on incorporation of each deoxynucleoside triphosphate independently showed that the poly(A) segment of mRNA, to which the oligo(dT) primer anneals, is not preferentially transcribed into DNA. In fact, when the long oligo(dT) primer is used, there is no preferential incorporation of dTTP. This result indicates that little or none of the poly(A) portion is transcribed, and that transcription takes place on the messenger sequence of 14S RNA. This result could be explained if the large primer covers up most of the poly(A) portion of the mRNA and prevents its transcription.

Antibody diversity is determined by genes for the variable portion of immunoglobulin molecules. The measure of frequency of those genes could best be achieved with radioactive DNA complementary to that half of the mRNA that codes for the variable part of the light chain (5′-half of the mRNA). Since initiation with oligo(dT) takes place at the 3′-end of the light chain mRNA, the DNA produced will always contain sequences homologous to the genes for the constant part of light chains. However, if initiation of cDNA synthesis could take place within the mRNA molecule, and near the beginning of the variable part of the mRNA (coding for amino-acids 109–214 of MOPC 41), the cDNA made would be exclusively complementary to the variable genes. If a cluster of either C, G, or U existed within this region of the mRNA, it could anneal to a complementary oligodeoxynucleotide primer and therefore allow "internal initiation" of cDNA synthesis. The two proline residues in positions 119 and 120 of the MOPC 41 light chain, together with the adjacent amino acids (13), correspond to a cluster of between 6 and 12 cytidine residues in the mRNA. When the three possible primers [oligo(dC), oligo(dA), and oligo(dG)] were tested, which should not anneal to poly(A), only oligo(dG) primed for DNA synthesis. The synthesis obtained with oligo(dG) primer presumably results from annealing of that primer to an oligo(rC) sequence within the mRNA molecule. It remains to be shown that, if as predicted above, the product resulting from initiation with oligo(dG) is exclusively complementary to the variable portion of the light chain mRNA.

It is of interest that in the only two cases where a purified eukaryotic mRNA has been tested as template for cDNA synthesis, with hemoglobin mRNA (4–6) and now with light chain mRNA, the results have been positive. Since all mRNAs seem to have poly(A) sequences, with the possible exception of histone mRNA (14), it is possible that all mRNAs could be potential templates for cDNA synthesis with an oligo(dT) primer and with RNA-dependent DNA polymerase. It is tempting to speculate that this might not simply be a fortuitous coincidence but that it might have important biological implications, even though the presence of RNA-dependent DNA polymerase in normal tissues is not unequivocally documented (15).

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