Methods in CANCER RESEARCH

VOLUME XII

Edited by Harris Busch

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BAYLOR COLLEGE OF MEDICINE
HOUSTON, TEXAS

VOLUME XII

ACADEMIC PRESS New York San Francisco London 1976
A Subsidiary of Harcourt Brace Jovanovich, Publishers

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 66-29495

ISBN 0-12-147672-3

PRINTED IN THE UNITED STATES OF AMERICA

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Preface

Among the many areas of viral oncology, that of the presence of "reverse transcriptase" in tumor cells has been of particular interest not only because of the recent award of the Nobel Prize to Temin and Baltimore, but because of the many studies from Spiegelman's group that have indicated human tumors may contain special enzymes of this type. The chapter of Gallo's group on this subject is especially timely. The new methods for mapping and sequencing DNA have been extensively applied to tumor virus DNA; the chapters of Griffin and Fried and Wu *et al.* on these important topics are particularly valuable at this time. The identification and evaluation of tumor viruses in Dmochowski's laboratory has emerged from application of many methods discussed in their chapter on "Electron Microscopy of Viruses."

The subject of isozymes has in the past yielded valuable information with respect to the cancer problem. Methods for studies on isozymes of carbohydrate enzymes are reviewed by Sato and Sugimura in the section "Differentiation of Cancer Cells." Hollenberg and Cuatrecasas and Clark *et al.* present important methods for evaluation of hormone receptors including affinity chromatography and a variety of other methods for characterization and quantitation of these important receptors. Needless to say, this subject has become increasingly significant in view of the recent developments with respect to various clinical modalities of therapy of breast cancer based upon the presence or absence of estrogen receptors.

The continuing evolution of methodology in the cancer problem and the more incisive techniques available now by comparison with those available when the series was initiated ten years ago provide the hope that in a relatively short time more specific and usable information on cancer cells will soon become available.

HARRIS BUSCH

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MOLECULAR VIROLOGY

CHAPTER I

REVERSE TRANSCRIPTASE OF RNA TUMOR VIRUSES AND ANIMAL CELLS

M. G. SARNGADHARAN, H. S. ALLAUDEEN, AND R. C. GALLO

I. Introduction

Indications that the replication of RNA tumor viruses might involve DNA synthesis (Temin, 1964a; Bader, 1965) led Temin to postulate that these viruses replicate via a DNA "provirus" proposed to arise by reverse transcription of viral RNA (Temin, 1964b). Subsequently, an RNA-dependent

DNA polymerase (reverse transcriptase) activity was discovered in RNA tumor viruses (Baltimore, 1970; Temin and Mizutani, 1970; for reviews, see Temin and Baltimore, 1972; Sarin and Gallo, 1974a; Green and Gerard, 1974). Reverse transcriptase is distinct from the DNA polymerases of cells. It is coded for by the viral genome (Verma *et al.*, 1974a), and it is necessary for the infection of cells by these viruses in order to catalyze the transcription of the viral RNA into DNA (Hanafusa and Hanafusa, 1971). It appears that all infectious RNA tumor viruses contain this polymerase (Gallo, 1972); a few other viruses, closely related to RNA tumor viruses, also contain this or a very similar polymerase. These viruses include the visna virus (Schlom *et al.*, 1971), a virus responsible for some neurological diseases of sheep, and the so-called A particles. The latter include the intracytoplasmic A particles and intracisternal A particles. Both particles are found only within the cell; although their exact origin and function are not fully understood, there is evidence to indicate that intracytoplasmic particles are precursors to type B viruses (Wilson *et al.*, 1974; Yang and Wivel, 1974).

There are common biochemical properties of reverse transcriptase from different viruses. For example, they have a relatively high affinity for certain synthetic primer-templates such as $(dT)_{\sim 15} \cdot (A)_n$ (Goodman and Spiegelman, 1971; Robert *et al.*, 1972; Wells *et al.*, 1972) and $(dG)_{\sim 15} \cdot (C)_n$ (Baltimore and Smoler, 1971) and a relatively poor affinity for the synthetic DNA homopolymer templates (Robert *et al.*, 1972). Unlike eukaryotic cellular DNA polymerases, they also can catalyze transcription of heteropolymeric regions of viral 70 S RNA and various mRNAs with little apparent specificity (Duesberg *et al.*, 1971a; Bhattacharyya *et al.*, 1973; Faras *et al.*, 1972; Verma *et al.*, 1972; Kacian *et al.*, 1972; Ross *et al.*, 1972; Chen *et al.*, 1973; Berns *et al.*, 1973). Several other natural RNAs were examined for their template activity. These include tobacco mosaic virus RNA, influenza virus RNA (Duesberg *et al.*, 1971a,b), polio RNA (Faras *et al.*, 1972; Taylor *et al.*, 1973), R17 27 S RNA, total RNA from HeLa cells, and HeLa tRNA (Faras *et al.*, 1972). None of these molecules was substantially transcribed by the reverse transcriptase. Reverse transcriptases from different mammalian type C viruses are in general 4.5 S in size (Leis and Hurwitz, 1972; Lewis *et al.*, 1974a), show much more activity in the presence of Mn^{2+} than with Mg^{2+} (when synthetic primer-templates are used), and are related by immunological properties, although in general they can be distinguished from one another by the same assays (Parks *et al.*, 1972; Scolnick *et al.*, 1972a,b; Nowinski *et al.*, 1972; Todaro and Gallo, 1973; Yaniv *et al.*, 1974). Reverse transcriptase from virus derived from one species can be distinguished from reverse transcriptase derived from another species, and often between two different viruses derived from the same species.

In recent years, reverse transcriptase has been used as a sensitive means for detecting and quantitating oncornaviruses, for making radiolabeled DNA "probes" from various RNA molecules to use in molecular hybridization experiments, and as a virus marker, i.e., an indication that a cell not shown to be releasing virus has either been infected by virus or is synthesizing viral proteins through expression of endogenous viral information.

Many of the above-mentioned biological and biochemical properties and some of the uses of reverse transcriptase will be elaborated on in later sections. Some of the variations in properties of the enzyme from different types of RNA tumor viruses will also be described. The major objectives of this report, however, are to provide: (1) methods of assay of reverse transcriptase from crude virus preparations; (2) purification of reverse transcriptase from extracellular virus; and (3) purification of reverse transcriptase from cells that have expressed viral information. This will necessitate a discussion of the biochemical properties of this enzyme, especially those that distinguish it from cellular DNA polymerases. Every approach described in the literature will not be reviewed; also this report does not include further discussions on biological studies nor any discussions on the mechanisms involved in catalyzing RNA-directed DNA synthesis.

II. Reverse Transcriptase Reaction in Disrupted Virions

A. Purification of Virus

For detection and analysis of virus-associated enzyme reactions, it is essential to use virus preparations as free of cellular contaminants as possible. Several procedures for purification of viruses were described. Generally a large quantity of RNA tumor virus can be obtained by two methods. Either viruses are isolated from the supernatant medium of infected cells which are producing virus particles, or viruses are propagated in a susceptible animal and virus particles are isolated from body fluid. For example, avian sarcoma virus is grown in tissue culture by the following method: secondary cultures of susceptible chicken embryo fibroblast cells are infected with about 5 to 10×10^5 focus-forming units of an avian sarcoma virus. When 30–50% of the cells in a culture become transformed, the medium is collected once or twice a day from each plate, and new medium is added to the cultures. The collected medium is stored at $-70°C$ when purification of virus particles does not proceed immediately.

Generally virus preparations can be concentrated and at least partially freed from cellular contaminants by a combination of differential centrifugation in glycerol and sucrose density gradients. The particles banding at a density of 1.14–1.17 gm/ml in a sucrose equilibrium density gradient are

generally suitable. As a representation, one such method (Mizutani *et al.*, 1974) is described below. It is preferable to proceed with purification of virus without freezing and thawing of virus-containing fluid, as this may lead to considerable loss of virus. For example, when avian myeloblastosis virus (AMV) in plasma was obtained frozen, large amounts of virus aggregated and hindered purification; losses of up to 80% of the virus were observed (Leis and Hurwitz, 1974). Pooled media containing avian sarcoma virus are thawed and centrifuged at 6000 *g* for 20 minutes to remove non-viral metarials. Virus is then pelleted by centrifugation for 1 hour in a Spinco No. 30 rotor at 25,000 rpm. The virus pellet is resuspended in 10 m*M* Tris-HCl buffer (pH 7.5) and is dispersed with a glass Potter–Elvehjem homogenizer. Concentrated virus is placed on a layer of 15% (w/w) sucrose in 10 m*M* Tris-HCl, pH 7.5, containing 1 m*M* EDTA and centrifuged at 25,000 rpm for 1 hour in a Spinco SW 25.1 rotor on to a cushion of 65% (w/w) sucrose. The virus band is collected from the interphase between 15 and 65% sucrose and further purified by equilibrium 20 to 65% (w/w) sucrose density gradient centrifugation in a Spinco SW 50.1 rotor at 35,000 rpm for 2 hours. The virus band at a density of 1.16 gm/cm^3 is collected and is used for DNA polymerase assay after disruption.

B. Assay System

The assay measures the incorporation of deoxyribonucleotides into acid-insoluble material. The conditions generally employed in our laboratory are as follows: the endogenous reaction mixture in a total volume of 50 µl contains 50 m*M* Tris-HCl (pH 7.5 for mammalian type C viruses and pH 8.3 for avian viruses), 5 m*M* DTT, 1 m*M* Mn^{2+} for mammalian viruses

TABLE I
Substrates for Reverse Transcriptase Reactions

	Nucleotide substrates	
Primer-template	Nonradioactive (80 µ*M*)	^{3}H-Radiolabeled (5–10 µ*M*)
Endogenous	dATP, dGTP, dCTP	dTTP
$(dT)_{\sim 15} \cdot (A)_n$	—	dTTP
$(dT)_{\sim 15} \cdot (dA)_n$	—	dTTP
$(dG)_{\sim 15} \cdot (C)_n$	—	dGTP
Viral 70 S RNA	dATP, dCTP, dTTP	dGTP[a]

[a]Viral 70 S RNA contains (A) -tracts and, if measurement of heteropolymeric DNA synthesis is critical, the radiolabel used should *preferably be dGTP or dCTP.*

or 10 m*M* Mg^{2+} for avian type C viruses, 60–100 m*M* K^+, 80 μ*M* each of dGTP, dATP, and dCTP, 5–10 μ*M* [^{3}H]dTTP (approximately 6000 cpm/pmole), 0.04% Triton X-100, and disrupted virus. For reactions with exogenous templates and purified enzyme preparations, the appropriate primer-template is added to the above system at a final concentration of 25 μg/ml and the nucleotide composition is replaced as indicated in Table I. When the concentration of enzyme protein is low, addition of bovine serum albumin (50 μg/ml) seems to be beneficial. The reaction mixture is incubated at 37°C for the appropriate time, and the reaction is terminated by the addition of 50 μg of yeast tRNA (10 μl) and about 3 ml of 10% trichloroacetic acid (TCA) containing 0.02 *M* sodium pyrophosphate. The precipitate is kept in ice for at least 10 minutes, then collected on a nitrocellulose filter* (0.45 μm, Millipore) presoaked in 5% TCA containing 0.02 *M* sodium pyrophosphate. A convenient procedure is to use a multisample filter box† connected to a laboratory vacuum line which enables one to filter several samples simultaneously. The filters are rinsed thoroughly with 5% TCA containing pyrophosphate, and finally with 70% ethanol. The washed filters are removed and dried under a heat lamp; their radioactivity is determined by scintillation counting.

C. Reaction with Endogenous Primer-Templates

The reverse transcriptase of RNA tumor viruses exists as a complex with the genomic RNA within the virion core (Gerwin *et al.*, 1970; Coffin and Temin, 1971; Robinson and Robinson, 1971; Stromberg, 1972; Wu *et al.*, 1973); this can be demonstrated by isolating the core structures by equilibrium sucrose gradient centrifugation after dissociating the virion envelope by mild detergent treatment. One such experiment using AMV is represented in Fig. 1. In this complex, the enzyme can transcribe the endogenous RNA when provided with the appropriate substrates and cofactors (Temin and Mizutani, 1970; Baltimore, 1970). Endogenous DNA synthesis is carried out by disrupting the virus with nonionic detergents and incubating with all the four deoxynucleoside triphosphates and either Mg^{2+} or Mn^{2+} in a suitable buffer system. In this reaction, one or all of the nucleotides used could be radiolabeled, enabling one to obtain labeled DNA copies of the viral genome. Such labeled DNA "probes" are extremely useful in molecular hybridization studies looking for viral RNA or DNA sequences in a test

*Nitrocellulose filters of 0.45 μm pore size are commercially available from Millipore Corporation, Bedford, Massachusetts (Cat. No. HAWP-2500). Comparable material is also available from Gelman Instrument Company and Schleicher and Schuell.

†These multiple sampling manifolds, which can filter 12 or 30 samples at a time, are available from Millipore Corporation.

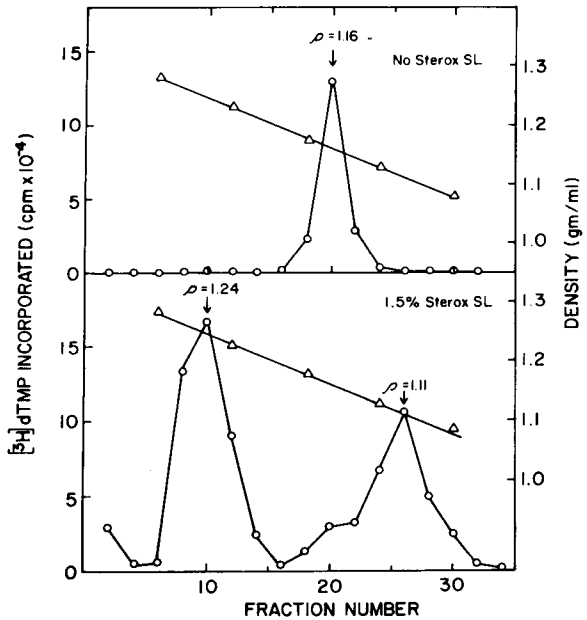

FIG. 1. Separation of core structures of avian myeloblastosis virus by equilibrium sucrose density gradient centrifugation. Avian myeloblastosis virus, purified from infected chicken plasma, was incubated in ice for 30 minutes with or without 1.5% Sterox SL in a total volume of 3.25 ml and layered on a 20–60% (w/w) sucrose gradient (34 ml) in 10 m*M* Tris-HCl (pH 7.5)–1 m*M* EDTA and centrifuged for 18 hours at 27,000 rpm using a Spinco SW27 rotor. About 35 equal fractions were collected from the bottom of the tube, and aliquots were assayed for reverse transcriptase activity after preincubation at 37°C with 0.25% Triton X-100 and 0.5 *M* KCl for 10 minutes. Final concentration of Triton X-100 and KCl in the assay were 0.05% and 0.1 *M*, respectively. The primer-template used was $(dT)_{\sim 15} \cdot (A)_n$. See Section II,D and Table I for assay conditions. ○—○, reverse transcriptase activity; △—△, density.

system (Hehlmann *et al.*, 1972; Kufe *et al.*, 1972, 1973a; Gallo *et al.*, 1973a; Miller *et al.*, 1974).

1. Reaction Requirements

a. Disruption of Viruses. The common method is to treat the RNA tumor viruses with a nonionic detergent, such as Nonidet P-40 (NP-40), Triton X-100, Sterox SL, or other agents that either alter the permeability of the virus or disrupt their outer envelope since the virions are impermeable to even small molecules, such as nucleoside triphosphates. Murine and avian leukoviruses seem to differ in the amount of detergent required for maximum endogenous reverse transcriptase activity. Murine leukemia virus preparations have maximal activity after treatment with about 0.04% Triton X-100; excess of detergent leads to marked inhibition of endogenous