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Biochemistry

Cell-free synthesis of the precursor polypeptide for avian myeloblastosis virus DNA polymerase

(group-specific antigen/DNA nucleotidyltransferase/polyprotein/genetic map/enzyme processing)

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ABSTRACT **High molecular weight RNA (35S) isolated from avian myeloblastosis virus directs the cell-free synthesis of two prominent polypeptides of 180,000 and 76,000 molecular weight. The latter polypeptide has previously been identified as the precursor to the group-specific antigens of the virus ("gag" proteins) [Vogt, V. M., Eisenman, R. & Diggelmann, H. (1975) *J. Mol. Biol.* 96, 471-493]. Two-dimensional tryptic peptide analyses of the [^{35}S]methionine-labeled peptides demonstrate that the 180,000-dalton product is a polyprotein that can account for all the peptides of the avian myeloblastosis virus DNA polymerase (DNA nucleotidyltransferase, EC 2.7.7.7.) and those of the gag viral proteins. This is direct confirmation of the genomic order of the viral structural genes, placing the polymerase gene adjacent to the 5′-proximal *gag* gene of the virus. Furthermore, our findings suggest that the primary polymerase gene product is the β subunit of the enzyme. These results are discussed in relation to the proposed structural gene map for the avian retraviruses and suggest a model for the *in vivo* processing of the viral polymerase.**

The structural proteins of the retraviruses are synthesized *in vivo* as large polypeptide precursors which are cleaved subsequently by proteolysis to the structural proteins characteristic of the mature virus particle (1, 2). Similarly, we, as well as others, have demonstrated that the β subunit of the avian myeloblastosis virus (AMV) DNA polymerase (DNA nucleotidyltransferase, EC 2.7.7.7) is the precursor to the α subunit and that the α subunit is derived proteolytically from the β subunit (3-5). In the present communication we demonstrate that the β subunit of AMV DNA polymerase and the group-specific antigen ("gag") structural proteins of the virus are synthesized as a 180,000 molecular weight (M_r) polyprotein in a cell-free translational system. These two proteins can account for all of the [^{35}S]methionine-labeled peptides present in the polyprotein. Taken in conjunction with previously reported observations (1, 2, 6-10), these results directly demonstrate the gene for AMV DNA polymerase is adjacent to the 5′-proximal *gag* gene. This defines the structural gene order within the viral genome and suggests a scheme for the processing of the AMV DNA polymerase *in vivo*.

MATERIALS AND METHODS

Virus Preparations. AMV was obtained from the plasma of infected chicks provided through contract number N01CP33291 under the Virus Cancer Program of the National Cancer Institute. The virus was concentrated and purified as described previously (11).

Extraction of Viral RNA. Viral RNA was prepared from fresh viral pellets and extracted as described previously (12). Viral RNA was fractionated on sucrose gradients and the 70S RNA fraction was precipitated with ethanol; pellets were dissolved in buffer and heated to 90° for 3 min, and the RNA was refractionated on sucrose gradients. The 35S RNA peak was precipitated with ethanol and used for cell-free translation. This 35S viral RNA was completely free of primer and 70% of the RNA was shown to be full size (35S) by electrophoresis on agarose slab gels containing methyl mercury (13).

Preparation of Goat IgG Directed to AMV DNA Polymerase. Goat anti-AMV DNA polymerase IgG was obtained from the Virus Cancer Program of the National Cancer Institute.

Preparation of Authentic [^{35}S]Methionine-Labeled Rous Sarcoma Virus DNA Polymerase. Chick embryo fibroblasts from primary cultures were trypsinized and infected with Bryan high-titer Rous sarcoma virus (BH-RSV) and grown in roller bottles at 39° in Eagle's minimal essential medium containing 5 mM sodium pyruvate, glucose at 2 g/liter, 5% fetal bovine serum, 10% tryptose phosphate broth (Difco), penicillin at 62 μg/ml, and streptomycin at 135 μg/ml. High density cultures were then labeled for two consecutive 12-hr periods with [^{35}S]methionine at 10 μCi/ml (Amersham, 600–800 Ci/mmol) in medium containing $^1/_{15}$ the normal concentration of methionine. After clarification of the medium by low-speed centrifugation (5000 × *g* for 10 min), the virus was pelleted by centrifugation at 40,000 rpm for 3 hr at 4° in a Beckman SW 41 rotor. Viral pellets were suspended and vortex mixed in buffer A (0.01 M Tris·HCl, pH 7.2/0.1 M NaCl/0.6 mM EDTA/0.67% NP-40 nonionic detergent/0.67% sodium deoxycholate/0.8 M KCl) at room temperature. Debris was removed by centrifugation at 16,000 × *g* for 10 min at 4°. The supernatant fluid was diluted with two volumes of buffer B (50 mM Tris·HCl, pH 7.8/0.3 M NaCl/2% Triton X-100) and applied to a 6-ml column of goat anti-AMV DNA polymerase IgG coupled to Sepharose 4B. Prior to loading, the column was equilibrated with buffer C (50 mM Tris·HCl, pH 7.8/0.3 M NaCl/bovine serum albumin at 10 mg/ml/2% Triton X-100) and excess serum albumin was washed from the column with buffer B. This procedure greatly reduced nonspecific binding of proteins to the column. The column was washed to background radioactivity with buffer B containing no Triton X-100. The bound [^{35}S]methionine-labeled viral proteins were quantitatively eluted at 60° with 50 mM NH_4OH and 0.2% sodium dodecyl sulfate. The eluate was lyophilized and dissolved in sample buffer for preparative electrophoresis on sodium dodecyl sulfate/polyacrylamide slab gels (14). Authentic [^{14}C]-formaldehyde-labeled AMV polymerase was used as a marker for the preparative gel (15). Polymerase polypeptides were extracted and trypsinized as described previously (16).

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Abbreviations: AMV, avian myeloblastosis virus; gag, group-specific antigens of the AMV virus; M_r, molecular weight; BH-RSV, Bryan high-titer Rous sarcoma virus.

Cell-Free Translation and Product Identification. AMV RNA was translated in the mRNA-dependent reticulocyte lysate prepared according to the procedures of Pelham and Jackson (17). Reactions contained, in a final volume of 25 μl: 10 μl of lysate, 135 mM KCl, 1.75 mM Mg acetate, 1 mM ATP, 0.4 mM GTP, 600 μM spermidine (free base), 8 mM creatine phosphate, creatine kinase (155 units/mg, Sigma) at 8 μg/ml, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.6, each amino acid except methionine at 25 μM, 2 mM dithiothreitol, 10–15 μCi of [^{35}S]methionine (400–800 Ci/mmol, Amersham), deacylated rabbit liver tRNA at 55 μg/ml (kindly supplied by Dolf Hatfield), and 0.5–2.0 μg of AMV RNA. Reaction mixtures were incubated at 37° for 1 hr and assayed for incorporation of radioactivity into material precipitable by hot trichloroacetic acid as described (16). Samples were run on sodium dodecyl sulfate/polyacrylamide slab gels (14). Tryptic product analyses were performed on polypeptides fractionated on preparative polyacrylamide gels, extracted and digested with trypsin as described (16). Peptide maps were run on cellulose thin-layer plates (Avicel-Merck) according to Pawson *et al.* (6). Thin-layer plates were fluorographed according to Bonner and Stedman (18), on XR-5 Kodak film at −80° for 2–24 hr.

RESULTS

***In Vitro* Translation of 35S AMV RNA.** Addition of 35S AMV RNA to an mRNA-dependent rabbit reticulocyte lysate cell-free system resulted in the synthesis of a prominent polypeptide of 76,000 M_r (Fig. 1*B*). This product has been previously identified as the precursor to the gag structural proteins characteristic of the avian RNA tumor viruses (1). An additional component synthesized in this system in significant amounts was a polypeptide with a 180,000 M_r (Fig. 1*B*). This latter polypeptide was not synthesized in detectable amounts in response to AMV RNA sedimenting at 30 S or less (Fig. 1*A*). The reasons for this discrepancy are unclear, but the most likely explanation is the physical trapping of degraded genomic RNA in the lower molecular weight fractions of the gradient. Furthermore, the rate of synthesis of the 180,000 M_r polypeptide was $1/10$ to $1/20$ that of the 76,000 M_r gag polypeptide as judged by [^{35}S]methionine incorporation.

Tryptic Peptide Analysis of Cell-Free Products. The [^{35}S]methionine peptide maps of the isolated cell-free products are given in Fig. 2. As can be seen, the majority of peptide spots characteristic of 76,000 M_r polypeptide are found in the 180,000 M_r polypeptide (Fig. 2 *A* and *B*). Several peptides found in the 180,000 M_r polypeptide are not present in the 76,000 M_r polypeptide. The proposed structural gene map of the avian viral genome (7, 8) suggested that the extra peptides seen in the high molecular weight product synthesized in the cell-free system might represent those present in the viral polymerase. Fig. 2*C* shows the peptide pattern of purified DNA polymerase of BH-RSV; the α and β chains of the polymerase were obtained from a preparative sodium dodecyl sulfate/polyacrylamide gel of partially purified [^{35}S]methionine-labeled polymerase (Fig. 3). Earlier work by Gibson and Verma (5) has shown extensive homologies in the peptide maps for AMV and Rous sarcoma virus DNA polymerases. Practically all the major [^{35}S]methionine-labeled tryptic peptides found in the authentic polymerase (Fig. 2*C*) were also found in the tryptic pattern of the 180,000 M_r polypeptide. These peptides were not present in the tryptic patterns of the 76,000 M_r gag polypeptide (Fig. 2*B*). The gag-specific and polymerase-specific [^{35}S]methionine-labeled peptides present in the 180,000 M_r polypeptide

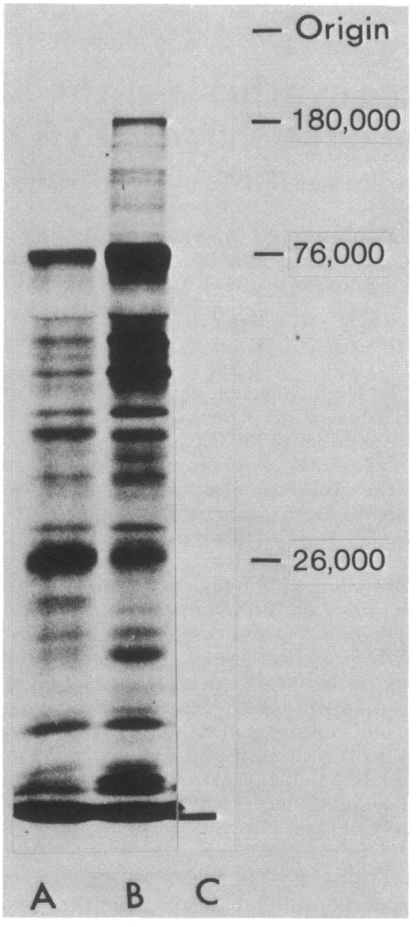

FIG. 1. Fluorograph of [^{35}S]methionine AMV polypeptides synthesized in an mRNA-dependent reticulocyte lysate and fractionated on a sodium dodecyl sulfate/8% polyacrylamide gel. The dried gel was fluorographed at −80° for 8 hr with Kodak XR-5 film. Synthesis was in response to: (*A*) 2 μg of 30S AMV RNA; (*B*) 2 μg of 35S AMV RNA; (*C*) no added RNA.

are indicated in the composite diagram in Fig. 2*D*. In addition to the 14 polymerase peptides, 19 of the 22 peptides characteristic of the 76,000 M_r polypeptide were accounted for in the tryptic pattern of the 180,000 M_r polypeptide. No peptides in the 180,000 M_r polypeptide were attributable to proteins other than the gag protein or viral polymerase.

DISCUSSION

Our results demonstrate that the *gag* and polymerase genes are adjacent on the avian oncornavirus genome because these two genes can be translated as a single polypeptide in a cell-free protein-synthesizing system in response to 35S AMV RNA. Taken in conjunction with previously published work (1, 2, 6–10), this would place the polymerase gene next to the 5'-proximal *gag* gene in the viral genome and define the structural gene order in the avian retraviruses as 5'-*gag-pol-env-src*(*leuk*)-poly(A)-3'. This is in agreement with the gene map proposed for these viruses (7–10). Our findings support similar results obtained by Jamjoom *et al.* (2) from *in vivo* pulse-chase experiments with Rauscher leukemia virus-infected cells; immunoprecipitation with monospecific antibodies suggested that the gag and polymerase determinants were present in a high molecular weight polypeptide (~200,000 M_r) synthesized in Rauscher leukemia virus-infected cells. Thus, the avian RNA tumor viruses would appear to be similar to murine RNA tumor

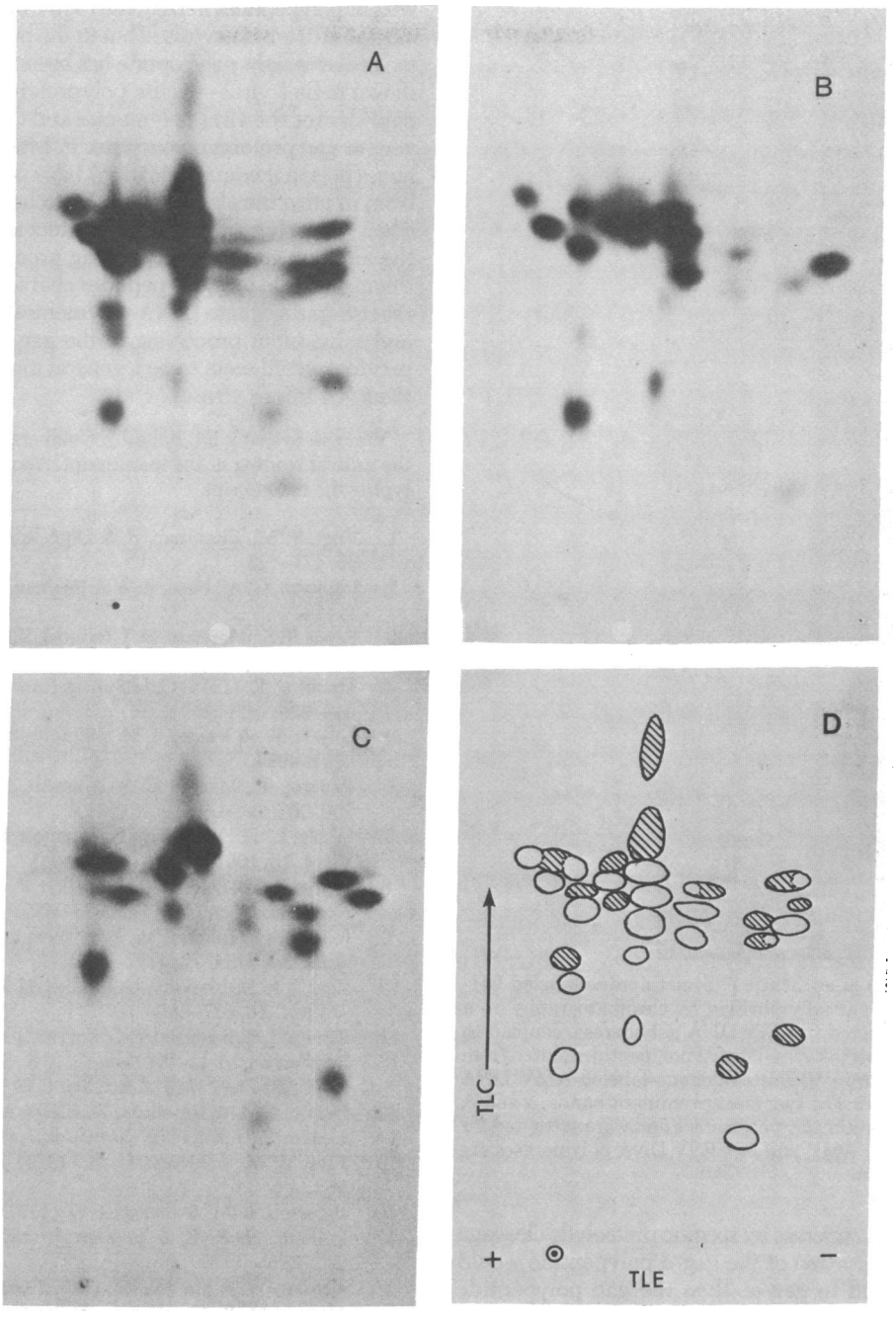

FIG. 2. Fluorograph of [^{35}S]methionine-containing tryptic peptides of the 180,000 M_r and 76,000 M_r polypeptides synthesized in the mRNA-dependent reticulocyte lysate in response to 35S AMV RNA, and of authentic BH-RSV DNA polymerase. TLC, thin-layer chromatography; TLE, thin-layer electrophoresis. The fingerprint analyses are (*A*) 180,000 M_r polypeptide; (*B*) 76,000 M_r polypeptide; (*C*) authentic BH-RSV DNA polymerase, both the α and β chains; and (*D*) a composite map of the polymerase-specific (hatched) and 76,000 M_r-specific (open) polypeptides found in the 180,000 M_r polypeptide.

viruses in the organization of the genome and the mode of viral polypeptide synthesis.

If the cell-free products can be taken as a measure of the steady-state amount of polymerase polypeptide and gag polypeptide, our results may explain the quantitative differences observed in the *in vivo* synthesis of these two viral components. In agreement with our observations, experiments by Arlinghaus and coworkers have demonstrated that $1/10$–$1/25$ as much polymerase precursor (180,000 M_r) as gag precursor (76,000 M_r) is present in the cell after *in vivo* labeling (2). Our data support the notion that the differential synthesis of gag and polymerase is mediated at the tranlational level, possibly by partial ribosome suppression of a terminator signal as proposed by Arlinghaus (2).

On the basis of the molecular weights of the gag-polymerase polypeptide (180,000 M_r) and the gag protein (76,000 M_r), we conclude that the primary gene product for the polymerase is the β subunit (98,000 M_r). This suggests the following processing models for the assembly of functional viral polymerase. In the first instance, the 180,000 M_r gag-β precursor polypeptide would be specifically cleaved to the gag polypeptide (76,000 M_r) and the β subunit of the polymerase (98,000 M_r). Subsequently, the β polymerase subunit would dimerize to the $\beta\beta$ form of the enzyme, which would then be converted to the $\alpha\beta$

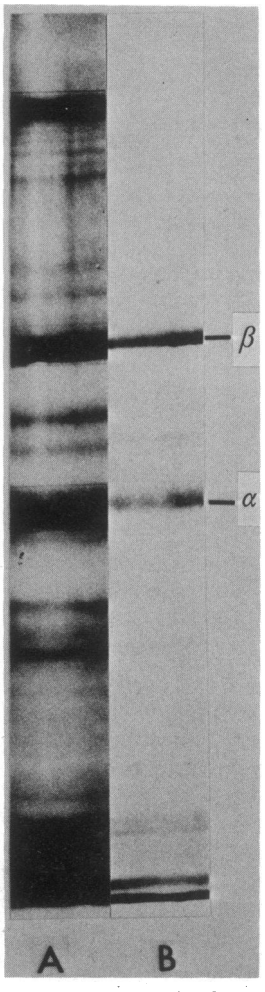

FIG. 3. Fluorograph of authentic [^{35}S]methionine-labeled BH-RSV DNA polymerase partially purified by chromatography on a column of goat IgG directed to AMV DNA polymerase, coupled to Sepharose 4B. (*A*) [^{35}S]Methionine-labeled polypeptides eluted from the antibody column; (*B*) [^{14}C]Formaldehyde-labeled AMV DNA polymerase polypeptides. The two most prominent bands, α and β, comigrate exactly with authentic polymerase and were extracted for tryptic peptide analysis. AMV and BH-RSV DNA polymerases are antigenically related (19).

configuration of the polymerase by specific proteolytic cleavage (19). Alternatively, a portion of the gag-β polypeptide would be specifically cleaved to gag-α, then the gag polypeptide would be removed by specific proteolytic cleavage from both the gag-α and gag-β precursors, leaving a mixture of the gag polypeptide and the α and β subunits of the polymerase. The α and β subunits would then dimerize to form the active polymerase. The $\beta\beta$ form of the enzyme has been isolated (20), suggesting the first processing scheme may be the correct one.

Other groups have reported the synthesis of high molecular weight polypeptides in response to RNA from avian (21, 22) and murine (10) oncornaviruses, but in the present instance the high molecular weight polypeptide has been fully characterized and shown to be a virus-specific polyprotein, containing the polypeptides for the viral polymerase and the group-specific antigens or gag proteins of the virus. E. Murphy and R. B. Arlinghaus (personal communication) have obtained similar results from *in vitro* translation of 35S Rauscher leukemia virus RNA. Their gag-polymerase *in vitro* product is also made in $\frac{1}{25}$ to $\frac{1}{20}$ the amount (in moles) of the gag precursors. It contains methionine-labeled tryptic peptides characteristic of p30 and the reverse transcriptase (DNA polymerase). Polyprotein synthesis and subsequent processing of the gag and polymerase polypeptides would seem to be a general mode in the replication of the RNA tumor viruses.

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