Proc. Natl. Acad. Sci. USA
Vol. 74, No. 10, pp. 4370–4374, October 1977
Biochemistry

Structural gene identification and mapping by DNA·mRNA hybrid-arrested cell-free translation

(hybrid plasmid PβG1/adenovirus 2/DNA restriction fragments/DNA·mRNA hybridization/cell-free protein synthesis)

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Communicated by Alexander Rich, August 8, 1977

ABSTRACT **We present a simple method for directly correlating structural gene sequences in DNA with their corresponding mRNAs. This is based upon the fact that mRNA hybridized with its complementary DNA will not direct the cell-free synthesis of a complete polypeptide. Full translational activity of the mRNA is recovered upon the heat melting of the hybrid. Utilizing the rabbit β globin clone PβG1, we demonstrate the application of hybrid-arrested translation for the identification of structural gene sequences within recombinant DNA molecules. In addition, the method is used to locate and order precisely several adenovirus 2 polypeptides within the viral genome.**

We have developed a method with which to analyze the relationship between DNA sequences, their corresponding messenger RNAs, and the proteins for which they code. This approach is based upon the observation that mRNA in hybrid form with its complementary DNA (cDNA) is not translated in eukaryotic cell-free systems, while heat dissociation of the hybrid reinstates complete translational activity (1). This observation has been extended to double-stranded DNAs by carrying out the hybridization reactions in a concentration of formamide that favors the formation of DNA·RNA hybrids but essentially prevents DNA·DNA reannealing (2–4).

Under optimized reaction conditions, it is demonstrated that the translation of β globin mRNA is reversibly arrested when total 9S rabbit globin mRNA is hybridized with the DNA of the recombinant plasmid PβG1. Hybrid-arrested translation (HART) was also used to map precisely specific protein-coding regions in restriction fragments of adenovirus 2 DNA. Thus, HART defines a simple method for the identification of genes or gene fragments encoding polypeptides in recombinant DNA molecules and a means for precisely mapping structural and regulatory sequences in DNA viruses.

MATERIALS AND METHODS

Wheat germ was kindly supplied by W. C. Mailnot, General Mills, Minneapolis, MN. Rabbits were purchased from the Charles River Breeding Laboratories, Boston, MA. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) and piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (Pipes), creatine (EC 2.7.3.2), and spermidine as the free base were purchased from Sigma Biochemicals. ATP, CTP, creatine phosphate, and dithiothreitol were from Boehringer Mannheim Biochemicals. Formamide, 99% (FX420), was purchased from Matheson, Coleman, and Bell. The restriction endonuclease *Hha* I from *Haemophilus haemolyticus* (ATCC 10014) was from New England Bio-Labs, Beverly, MA.

Preparation of DNA Restriction Fragments. The hybrid plasmid PβG1 containing the rabbit β globin DNA insert, and the S1 nuclease-excised β globin DNA fragment (4), were generous gifts of Argiris Efstratiadis. They were prepared under containment conditions consistent with the NIH Recombinant DNA Research Guidelines, Part II. The plasmid PβG1 was digested to completion with the restriction endonuclease *Hha* I in 6 mM Tris·HCl at pH 7.9/6 mM magnesium acetate/6 mM 2-mercaptoethanol at 37° (5). The digestion products were phenol extracted and stored as described below.

The purified *Bam*HI restriction fragments of adenovirus 2 DNA were very kindly provided by Michael B. Mathews.

Purification of DNA Restriction Fragments. Fractionated DNA restriction fragments were recovered from agarose gels by the method of Southern (6). Thereafter, phosphate was removed by dialysis against 10 mM Tris·HCl at pH 7.4/1 mM EDTA at 4°, and ethidium bromide was removed by Dowex 50 chromatography in 10 mM Tris·HCl at pH 7.4/0.1 mM EDTA. The DNA was concentrated by precipitation with 2.5 volumes of ethanol in the presence of 0.2 M sodium acetate at pH 5.5. The pellet was dissolved in 10 mM Tris·HCl at pH 7.4/0.1 mM EDTA. The solution was then adjusted to 0.1 M NaCl and extracted once with phenol equilibrated with 10 mM Tris·HCl at pH 7.4/0.1 mM EDTA and twice with chloroform/isoamyl alcohol (24:1, vol/vol). Finally, the DNA was precipitated with 2.5 volumes of ethanol in the presence of 0.2 M Na acetate at pH 5.5. The pellet was washed with 70% ethanol and the DNA was stored in water at 4°.

Preparation of Messenger RNA. Rabbit globin in RNA was prepared from salt-washed reticulocyte ribosomes (kindly supplied by Sherrill Adams) by the guanidine·HCl extraction procedure described previously (7) and enriched by oligo(dT)-cellulose chromatography (8). The 9S globin mRNA component was isolated on formamide/sucrose gradients (9) by Henry M. Kroneberg and kindly made available to us. Globin mRNA prepared by this method was shown by translation to contain both α and β globin mRNA species (Fig. 1, X). In contrast, it is noteworthy that globin mRNA prepared according to Efstratiadis *et al.* (10) is substantially depleted in translatable β globin mRNA (Fig. 1, Y).

Total cytoplasmic RNA from HeLa cells 24 hr after infection with adenovirus 2 was provided by Michael B. Mathews.

Conditions for Hybrid-Arrested Translation. For DNA excess hybridization, the appropriate amounts of linear DNA and mRNA were mixed in 2–3 μl of H_2O in a 1.5-ml polyethylene microcentrifuge tube, heated at 100° for 30 sec, quick chilled in a dry ice/methanol bath, and spun for 10 sec in a

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Abbreviations: HART, hybrid-arrested translation; cDNA, DNA complementary to RNA; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N'*-bis(2-ethanesulfonic acid).

Microfuge. The samples were then brought to a final volume of 25 μl containing 80% (vol/vol) deionized formamide (11), 10 mM Pipes, pH 6.4 at room temperature, and 0.4 M NaCl (2). The hybridization reactions were incubated at 48° for 2 hr and terminated by the addition of 200 μl of cold distilled water containing 25 μg of purified wheat germ tRNA. This amount of tRNA was previously shown not to inhibit cell-free translation or to contain detectable mRNA activity. The sample was then divided into two equal portions, one of which was retained in hybrid form, and the other was heated for 60 sec at 100° and quick-chilled in dry ice/methanol. Both the hybrid and heat-melted portions of the sample were then adjusted to 0.2 M sodium acetate at pH 5.5 and the nucleic acids were precipitated with 2.5 volumes of ethanol at −20° overnight or at −80° for 2 hr. The pellets were collected by centrifugation in a Microfuge at 12,000 × *g* for 10 min at 4°. The pellets were washed and recentrifuged twice with 0.75 ml of 70% (vol/vol) chilled ethanol and the residual ethanol was removed by lyophilization just prior to translation. The dried pellets were suspended in the appropriate volume of ice-cold distilled water and translated in either preincubated wheat germ extracts (12) or the mRNA-dependent reticulocyte lysate (13).

Wheat germ extracts were prepared as previously described by Roberts and Paterson (12) using 12 g of raw wheat germ in 28 ml of grinding buffer. Reaction mixtures contained, in a final volume of 25 μl/5 μl of preincubated wheat germ extract, 1 mM ATP, 0.4 mM GTP, 2.0 mM magnesium acetate, 600 μM spermidine (free base), 8 mM creatine phosphate, creatine kinase (Sigma, 155 units/mg) at 8 μg/ml, 85 mM potassium acetate, 25 mM Hepes at pH 7.6, 2 mM dithiothreitol, 25 μM each of the protein amino acids except methionine, and 10–15 μCi of [^{35}S]methionine (400–800 Ci/mmol, Amersham). Reaction mixtures were incubated at 23° for 2 hr.

Rabbit reticulocyte lysates were prepared by the method of Villa-Komaroff *et al.* (14) and treated with micrococcal nuclease according to Pelham and Jackson (13). Reaction mixtures contained in a final volume of 25 μl/10 μl of nuclease-digested reticulocyte lysate, 140 mM potassium acetate, 1.5 mM magnesium acetate, 500 μM spermidine (free base), 8 mM creatine phosphate, creatine kinase (Sigma, 155 units/mg) at 8 μg/ml, 20 mM Hepes at pH 7.6, 25 μM amino acids minus methionine, 2 mM dithiothreitol, and 10–50 μCi of [^{35}S]methionine (400–600 Ci/mmol). Reaction mixtures were incubated at 37° for 40 min. In both wheat germ extracts and reticulocyte lysates, incorporation of [^{35}S]methionine into protein was determined as described previously (12).

Analysis of Cell-Free Products. Rabbit α and β globin polypeptides were separated on phosphate-buffered sodium dodecyl sulfate/12% polyacrylamide gels as described by Weber and Osborn (15).

Adenovirus polyeptides were fractionated on sodium dodecyl sulfate/10–15% polyacrylamide gradient gels (16) stabilized by a 5–25% (wt/vol) sucrose gradient. In both cases gels were subjected to fluorography as described by Bonner and Laskey (17).

RESULTS

The hybrid plasmid PβG1, derived synthetically from rabbit β globin mRNA and the plasmid PMB9 (18), was used as a model system to characterize the hybridization reaction and demonstrate the application of HART for identification of recombinant DNA molecules containing specific structural gene sequences. Restriction fragments of adenovirus 2 DNA were used to show the application of this approach to the study of the fine structural and functional organization of protein coding regions within DNA viral genomes.

Translational Activity of mRNA Hybridized with DNA. Using the published rate values for DNA·RNA hybridization in high concentrations of formamide (2), the appropriate concentrations of DNA and mRNA were chosen such that hybridization would be completed within 30 min. In fact, hybridization times were extended up to 3 hr with no detectable loss in translational activity of mock-hybridized mRNA or mRNA that had been hybridized and then heat-melted. However, prolonged periods of hybridization (3–20 hr) resulted in variable recovery of mRNA translational activity.

The t_ms of DNA·mRNA hybrids were determined by correlating the reinstated translation of specific proteins with the temperature used to melt the hybrid. All of the hybrids thus far studied were melted and the mRNA was rendered available for translation by incubation at 80° for 1 min. Under our standard hybrid melt conditions [0.044 M Na^+ and 9% (vol/vol) formamide], the t_ms of individual hybrids ranged between 60° and 80° (data not shown).

Determination of the DNA/mRNA Sequence Ratio Required to Arrest Translation. To define the DNA sequence excess required to arrest mRNA translation and to demonstrate the utility of this technique for detecting structural gene sequences in recombinant DNA molecules, the rabbit β globin hybrid plasmid PβG1 was used. The parent plasmid PMB9 and the hybrid plasmid PβG1 were cleaved with the restriction endonuclease *Hha* I and hybridized in various ratios with a constant amount of total rabbit globin 9S mRNA. The hybrid and heat-melted mRNAs were translated in wheat germ extracts and the α and β globin polypeptides were separated on phosphate-buffered sodium dodecyl sulfate/polyacrylamide gels (Fig. 1). Messenger RNA that had been mock-hybridized in the absence of added DNA (Fig. 1, V and W) directed the synthesis of both α and β globin polypeptides in amounts equivalent to that of untreated mRNA (Fig. 1, X). Hybridization of the mRNA with increasing amounts of the digested PβG1 DNA (DNA/mRNA single-stranded sequence ratios were from 0.2 to 7.6) resulted in the progressive elimination of the β globin polypeptide with no alteration in α globin synthesis (Fig. 1, D to O). Full recovery of β globin mRNA translation was achieved upon heat melting the hybrid samples. At DNA/mRNA single-stranded sequence ratios greater than two, the synthesis of β globin could not be detected. Identical results were obtained with the purified S1 nuclease-excised β globin DNA (Fig. 1, P to U). When amounts of digested PMB9 DNA equivalent to the maximum input of PβG1 were hybridized with globin mRNA, no detectable alteration in α or β globin synthesis was detected (Fig. 1, B and C). Furthermore, the addition of up to 700 μg/ml of either native or denatured PMB9 DNA to the translation reaction did not result in a noticeable change in globin polypeptide synthesis (data not shown).

Structural Gene Arrangement in Adenovirus 2 DNA. Utilizing restriction fragments of adenovirus 2 DNA, HART was used to correlate DNA sequences with the messenger RNAs coding for specific viral proteins. Our results demonstrate that the restriction fragment *Bam* D (map coordinates 29.1 to 40.9) completely blocked the synthesis of adenovirus polypeptides III (penton base, molecular weight 85,000) and IIIa (molecular weight 66,000) as shown in Fig. 2, C and D. The *Bam* C fragment (map coordinates 40.9 to 59.0) blocked synthesis of the virus polypeptides II (hexon, molecular weight 120,000), III (penton base, molecular weight 85,000), V (minor core, molecular weight 48,500), pVI (molecular weight 24,000), and other unidentified minor constituents as shown in Fig. 2, E and

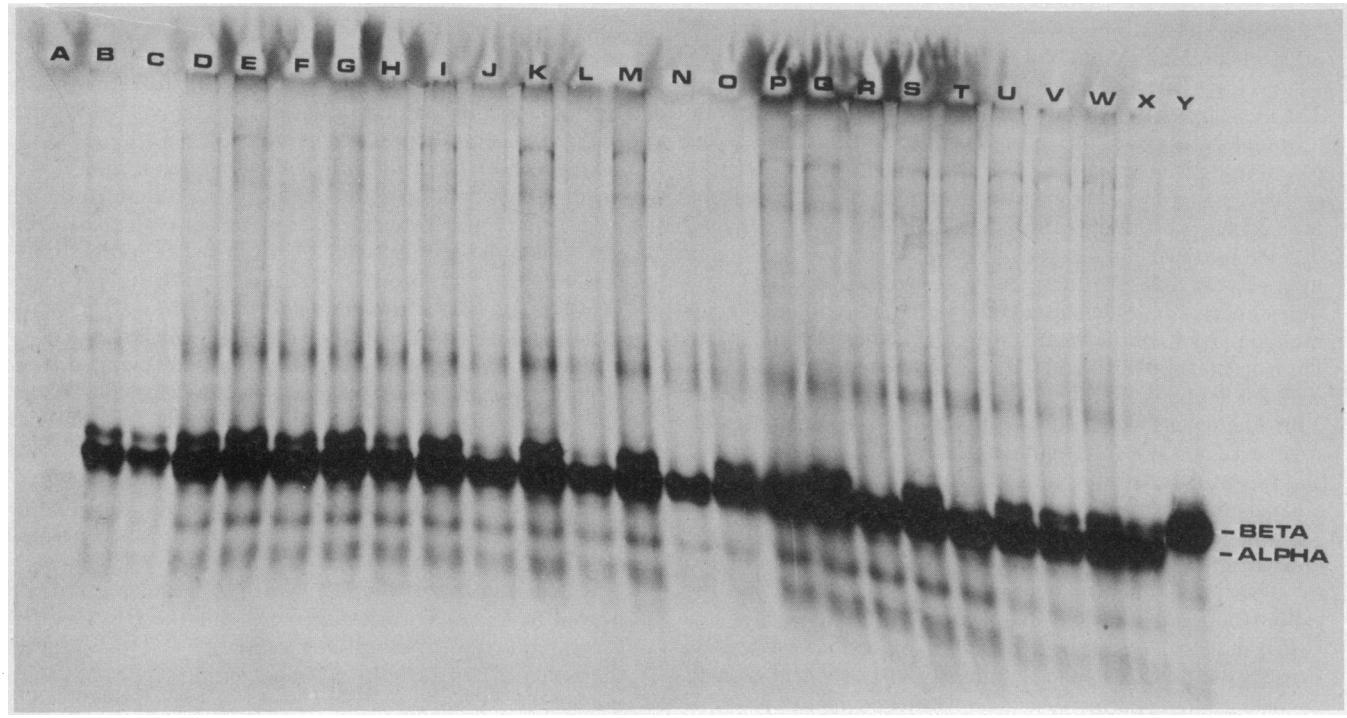

FIG. 1. Fluorograph of [^{35}S]methionine-labeled rabbit α and β globin polypeptides synthesized in a wheat germ cell-free system and separated on a phosphate-buffered sodium dodecyl sulfate/12% polyacrylamide slab gel. The dried gel was fluorographed at −80° for 15 hr using Kodak XR-5 film. Approximately 40,000 cpm was loaded in each slot. Samples contained the following: A, no added rabbit globin 9S mRNA; B through X contained 250 ng of total rabbit globin 9S mRNA and the following amounts of the appropriate DNA fragments. Reactions contained: B, RNA in hybrid conformation with 17 μg of PMB9 DNA; C, the heat melt of B; D, RNA in the hybrid conformation with 440 ng of PβG1 DNA; E, the heat melt of D; F, RNA in the hybrid conformation with 980 ng of PβG1 DNA; G, the heat melt of F; H, RNA in the hybrid conformation with 1.96 μg of PβG1 DNA; I, the heat melt of H; J, RNA in the hybrid conformation with 2.94 μg of PβG1 DNA; K, the heat melt of J; L, RNA in the hybrid conformation with 4.9 μg of PβG1 DNA; M, the heat melt of L; N, RNA in the conformation with 19 μg of PβG1 DNA; O, the heat melt of M; P, RNA in the hybrid conformation with 300 ng of the S1-excised β globin DNA; Q, the heat melt of P; R, RNA in the hybrid conformation with 900 ng of the S1-excised β globin DNA; S, the heat melt of R; T, RNA in the hybrid conformation with 1.5 μg of S1-excised β globin DNA; U, the heat melt of T; V, RNA alone in hybrid conformation; W, the heat melt of V; X, rabbit globin mRNA (total) untreated; Y, 200 ng of rabbit β globin mRNA. PMB9 is approximately 5000 base pairs; the β globin insert is approximately 580 base pairs and makes up 10% of the total hybrid plasmid PβG1 DNA (18).

F. In other gels, we have observed the blocked synthesis of pVII (molecular weight 20,000) following hybridization with *Bam* C fragment.

It is observed that the synthesis of polypeptide III (penton base) was arrested by both the *Bam* C and D restriction fragments. Furthermore, translation of *Bam* C-hybridized mRNA resulted in the appearance of a novel polypeptide that migrated ahead of polypeptide IV (fiber, molecular weight 62,000) and is indicated by the arrow in Fig. 2, E. The synthesis of this polypeptide was eradicated when the hybrid was heat melted (Fig. 2, F). It is noteworthy that this was the only peptide whose synthesis was dependent upon the formation of mRNA·DNA hybrids.

DISCUSSION

Nucleic acid hybridization reaction mixtures containing high concentrations of formamide effectively eliminate DNA·DNA reannealing and favor DNA·RNA hybridization (2, 3). These hybridization conditions were used to demonstrate that translatable mRNAs hybridized to double-stranded DNA containing their complementary sequences could be reversibly arrested for cell-free translation. We have determined that a DNA/mRNA single-stranded sequence ratio greater than two, hybridized under the conditions defined above, effectively blocked the synthesis of the polypeptide encoded by the mRNA. Heat melting of the hybrid reinstated the full translational activity of the sequestered mRNA. For convenience we have termed this procedure hybrid-arrested translation (HART).

Because the hybrid plasmid PβG1 has been shown to contain the complete coding sequence for rabbit β globin (19, 20), both the total linearized recombinant plasmid and the purified globin sequence thereof were used to demonstrate the application of the HART method as a general approach to the definition of structural gene sequences within recombinant DNAs.

The location of protein-coding regions within adenovirus 2 DNA has been defined previously by the translation of mRNAs selected by hybridization to specific restriction fragments of the viral DNA (21). Utilizing the adjacent restriction fragments *Bam* D (map coordinates 29.1 to 40.9) and *Bam* C (map coordinate 40.9 to 59.0) to arrest the translation of total cytoplasmic RNA from adenovirus-infected cells, we have defined the locations of six adenovirus polypeptides within the viral genome. The results agree with the locations determined by mRNA selection (21). The HART method demonstrates that *Bam* D includes sequences required for synthesis of polypeptides III and IIIa, and *Bam* C, sequences for II, III, V, pVII, and pVI. The tentative order of these polypeptides within the DNA is as follows: IIIa, III, (V, pVII, pVI), and II. This order is rationalized on the basis that synthesis of IIIa is arrested only by *Bam* D; III by both *Bam* D and *Bam* C; V, pVII, and pVI only by *Bam* C; and II by both *Bam* C and *Bam* A (data not shown

for the *Bam* A fragment, map coordinates 59.0 to 100). The order of V, pVII, pVI, and other unidentified polypeptides within the *Bam* C fragment is not known, but can be defined by further digestion of the *Bam* C DNA.

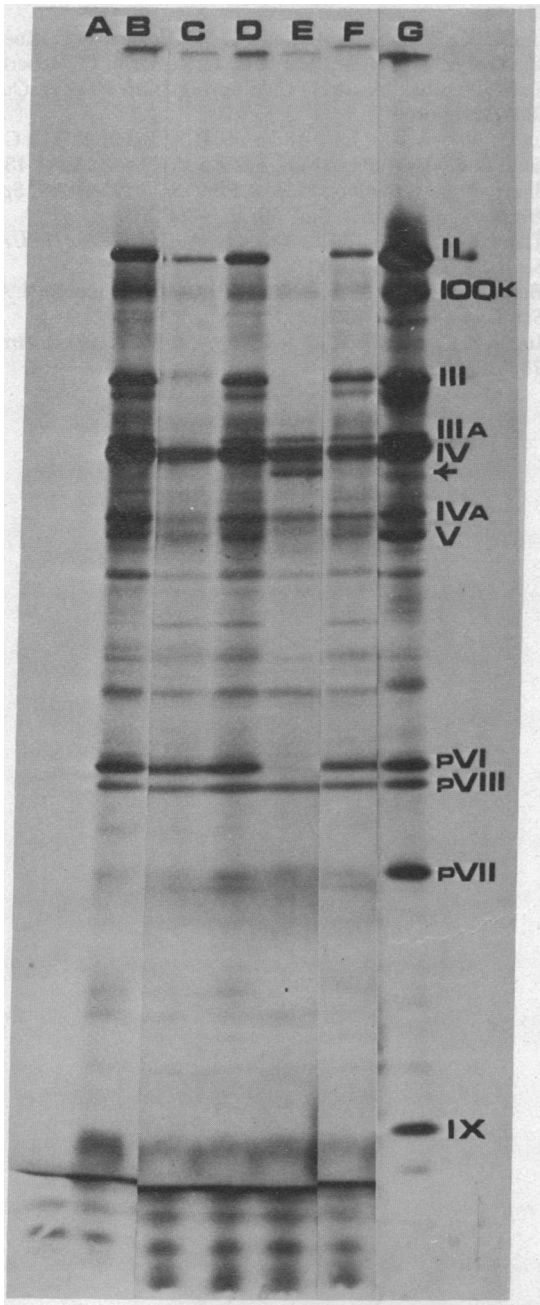

FIG. 2. Fluorograph of [^{35}S]methionine-labeled late adenovirus polypeptides synthesized in an mRNA-dependent reticulocyte lysate and fractionated on a sodium dodecyl sulfate/10–15% gradient polyacrylamide gel. The dried gel was fluorographed at −80° for 15 hr on Kodak XR-5 film. Approximately 80,000 cpm was loaded in each slot. The samples were: A, no added RNA; B, 500 ng of total cytoplasmic RNA from cells 24 hr after adenovirus 2 infection. Samples C through F all contained 500 ng of total cytoplasmic RNA from cells 24 hr after adenovirus 2 infection and 400 ng of the appropriate restriction fragment DNA: C, RNA in hybrid conformation with *Bam* D DNA (map coordinates 29.1–40.1); D, heat melt of C; E, RNA in hybrid conformation with *Bam* C DNA (map coordinates 40.1–59.0); F, heat melt of E; C, cytoplasmic extract of cells labeled with [^{35}S]-methionine 24 hr after adenovirus 2 infection. The map coordinates of the *Bam*HI digest of adenovirus 2 DNA are the unpublished data of C. Mulder and R. Greene.

The late RNA transcripts from that region of the adenovirus DNA contained within the *Bam* D and C fragments (29.1 to 59.0 map units) are transcribed in the rightward direction (22). Because both *Bam* D and C fragments eliminate the synthesis of penton (peptide III), the amino terminus of this protein appears to be within *Bam* D and the carboxy terminus within *Bam* C. It is noteworthy that RNA hybridized with *Bam* C DNA directs the synthesis of substantial quantities of a novel polypeptide smaller than penton, which is absent in hybrid reactions with *Bam* D DNA. Synthesis of this new component is eliminated upon heat melting of the *Bam* C·mRNA hybrid. These observations suggest that the novel polypeptide could be an amino-terminal fragment of penton resulting from premature termination of penton synthesis at the start of the mRNA·*Bam* C DNA hybrid region. This hypothesis can be checked by using different restriction fragments within the penton coding region and tryptic peptide analysis of the putative penton fragments. Confirmation would provide a method that permits the precise location of a viral polypeptide with respect to a given restriction fragment simply on the basis of the apparent molecular weight of the resultant novel polypeptide.

One of the major advantages of this direct biochemical mapping procedure is that structural gene sequences within DNA can be precisely located by using either the entire gene sequence or subfractions thereof to arrest the translation of specific mRNAs in a total unfractionated mRNA preparation. This obviates the problems associated with the purification of specific mRNAs.

There are numerous possible extensions of this approach which include: (*i*) the use of HART to determine the t_ms for specific mRNA·DNA hybrids to establish the precise temperature to R-loop a particular mRNA (23); (*ii*) the use of complementary DNA (cDNA) to study the gene organization and expression in certain RNA viruses; (*iii*) the use of cDNA to correlate abundance classes of mRNAs with particular proteins expressed in eukaryotic cells (1); (*iv*) the use of single-stranded DNAs from the appropriate restriction fragments to demonstrate strand switching of encoded information in double-stranded DNA viruses; (*v*) making amenable for direct biochemical mapping the DNA of large animal viruses such as those of the herpesvirus and poxvirus genera; (*vi*) the study of the function of leader sequences (24) and other untranslated portions of messenger RNAs.

While this work was in progress, Inglis *et al.* outlined a similar approach utilizing RNA·mRNA hybridization for gene mapping in the negative strand influenza A (fowl plague) virus (25).

We thank Michael B. Mathews and Argiris Efstratiadis for their enthusiastic support of this work. We are grateful to Lynna Hereford and Michael Rosbash for their helpful discussions and Gail Rondo for typing this manuscript. This work was supported in part by Grant 76-81653 to B.E.R. from the National Science Foundation.

1. Paterson, B. M. & Bishop, J. O. (1977) *Cell*, in press.
2. Casey, J. & Davidson, N. (1977) *Nucleic Acids Res.* **4**, 1539–1552.
3. Vogelstein, B. & Gillespie, D. (1977) *Biochem. Biophys. Res. Commun.* **75**, 1127–1132.
4. Hofstetter, H., Schamböck, A., van den Berg, J. & Weissmann, C. (1976) *Biochim. Biophys. Acta* **454**, 587–591.
5. Roberts, R. J., Breitmeyer, J. B., Tabachnick, N. F. & Myers, P. A. (1975) *J. Mol. Biol.* **91**, 121–123.
6. Southern, E. M. (1975) *J. Mol. Biol.* **94**, 51–71.
7. Strohman, R. C., Moss, P. S., Micou-Eastwood, J., Spector, D. &

Paterson, B. M. (1977) *Cell* **10,** 265–273.
8. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1408–1412.
9. Anderson, C. W., Lewis, J. B., Atkins, J. F. & Gesteland, R. F. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 2756–2760.
10. Efstratiadis, A., Kafatos, F. C., Maxam, A. M. & Maniatis, T. (1976) *Cell* **7,** 279–288.
11. Maniatis, T., Jeffrey, A. & van de Sande, M. (1975) *Biochemistry* **14,** 3787–3794.
12. Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2330–2334.
13. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67,** 247–257.
14. Villa-Komaroff, L., McDowell, M., Baltimore, D. & Lodish, H. F. (1974) in *Methods in Enzymology,* eds. Moldave, K. & Grossmann, L. (Academic Press, New York), Vol. 30, pp. 709–724.
15. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412.
16. Laemmli, U. K. (1970) *Nature New Biol.* **227,** 680–685.
17. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46,** 83–88.
18. Maniatis, T., Sim, G. K., Efstratiadis, A. & Kafatos, F. C. (1976) *Cell* **8,** 163–182.
19. Efstratiadis, A., Kafatos, F. C. & Maniatis, T. (1977) *Cell* **10,** 571–585.
20. Sim, G. K., Efstratiadis, A., Jones, C. W., Kafatos, F. C., Koehler, M., Kronenberg, H. M., Maniatis, T., Regier, J. C., Roberts, B. E. & Rosenthal, N. (1977) *Cold Spring Harbor Symp. Quant. Biol.* **42,** in press.
21. Lewis, J. B., Atkins, J. F., Anderson, C. S., Baum, P. R. & Gesteland, R. F. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1344–1348.
22. Sharp, P. A., Gallimore, P. H. & Flint, S. J. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39,** 457–474.
23. Thomas, M., White, R. L. & Davis, R. W. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2294–2298.
24. Berget, S. M., Moore, C. & Sharp, P. A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3171–3175.
25. Inglis, S. C., McGeoch, D. J. & Mahy, B. W. J. (1977) *Virology* **78,** 522–536.