JOURNAL OF VIROLOGY, July 1988, p. 2508–2511
0022-538X/88/072508-04$02.00/0

Vol. 62, No. 7

Functional Mapping of the Genome of the B19 (Human) Parvovirus by In Vitro Translation after Negative Hybrid Selection

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Received 14 December 1987/Accepted 22 March 1988

We have analyzed the coding capacity of B19 parvovirus transcripts by in vitro translation using the negative hybrid selection technique. Five different antisense oligonucleotides (18-mers) corresponding to different portions of the B19 genome were hybridized to RNA samples extracted from human erythroid bone marrow cells infected with B19 parvovirus in vitro, and RNase H was added to cleave specific B19 RNA molecules at selected sites. B19-specific translation products of these RNA samples were determined by immunoprecipitation. We localized the B19 nonstructural protein to the left-side transcript and the two capsid proteins to overlapping transcripts from the right side of the genome.

B19 parvovirus is the cause of the common childhood exanthem erythema infectiosum, a polyarthralgia syndrome in adults, and some cases of hydrops fetalis (N. S. Young, Semin. Hematol., in press). In individuals with underlying hemolysis, B19 infection results in transient aplastic crisis (2, 14), and in immunodeficient persons persistent B19 parvovirus causes chronic bone marrow failure (5). In vitro, B19 parvovirus inhibits erythroid colony formation (9, 16). The virus is highly restricted in its cellular host range and has been successfully propagated only in human erythroid bone marrow (11, 12).

Parvoviruses employ many strategies to amplify the coding capacity of their limited 5-kilobase genome (1). The transcription map of the B19 parvovirus in bone marrow cells has shown a pattern of RNA production more complicated than that described for other parvoviruses (10). There are nine polyadenylated, overlapping transcripts, all driven from a single promoter at the far left side of the genome (P_6). All but one of the transcripts are extensively spliced during processing. Variant polyadenylation signals are employed by some transcripts to terminate transcription in the middle of the genome.

Immunoblotting of infected sera (3) and immunoblotting and immunoprecipitation of cells infected with B19 in vitro (13) have shown two capsid proteins of 84 and 58 kilodaltons (kDa) and at least one nonstructural (NS) protein. By analogy with other parvoviruses and by extrapolation from the transcription map, B19 NS protein(s) should be encoded by transcripts from the left side of the genome and capsid proteins should be encoded by transcripts from the right side. Cotmore et al. (3) produced specific antisera directed against two peptides encoded by different halves of the B19 genome, the peptides synthesized by bacteria containing defined B19 sequences in expression vectors. Rabbit antibodies to a protein encoded between nucleotides (nt) 2897 and 3749 recognized the capsid proteins and antiserum to a protein encoded between nt 1972 and 2044 bound to three presumed NS proteins of 71, 63, and 52 kDa (3) (in bone marrow cells infected in vitro, a NS protein of 77 kDa has been consistently detected, with smaller peptides possibly representing degradation products [13]). To definitively determine which specific transcripts encode these protein products, we have used the method of negative hybrid selection to study translation in vitro.

The identity of an RNA species usually has been determined by either positive hybrid selection followed by translation or by "hybrid arrest of translation" (7). In hybrid selection, a short DNA fragment immobilized on a solid support is hybridized with a mixture of RNA species; specifically bound RNA can be eluted and used for in vitro translation. Hybrid selection has been commonly used, especially because of the advantage of obtaining specific RNA so highly purified that immunological identification of translation products is unnecessary. However, this method is technically unreliable and requires large amounts of RNA. In hybrid arrest of translation, failure to detect a protein product after hybridization of an RNA species with a specific DNA fragment has been interpreted as a block in ribosomal scanning of the mRNA molecule. However, Minshull and Hunt (8) have recently demonstrated that hybrid arrest probably occurs due to digestion of the hybridized regions with RNase H, which contaminates extracts used for in vitro translation. Addition of exogenous RNase H results in complete and specific arrest of translation by selective hydrolysis of RNA within RNA-DNA hybrids. (Hybrid arrest of translation is therefore more properly termed negative hybrid selection.) Synthetic oligonucleotides are so efficient in annealing to RNA that prehybridization is unnecessary (8), and, in contrast to restriction enzyme fragments, synthetic oligonucleotides can easily be prepared to complement defined RNA sequences. By using negative hybrid selection and in vitro translation, specific RNA molecules, even in complex RNA mixtures, can thus be cleaved with oligonucleotides and analyzed for coding and regulatory properties.

In vitro translation of B19 RNA from infected bone marrow cells. RNA was extracted from infected human erythroid bone marrow cells and translated in a cell-free, rabbit reticulocyte lysate system (Fig. 1). A 10-μg sample of total RNA (4.5 μl) was added to 35 μl of reticulocyte lysate (Promega Biotec, Madison, Wis.), 1 μl of methionine-free amino acid mixture (Promega), 5 μl of [^{35}S]methionine (10 μCi/μl; total, 50 μCi; specific activity, about 1,200 mCi/mmol; Amersham Corp., Arlington Heights, Ill.), 1 μl of 250 mM dithiothreitol, 1 μl of RNasin (40 U/μl; Promega), and

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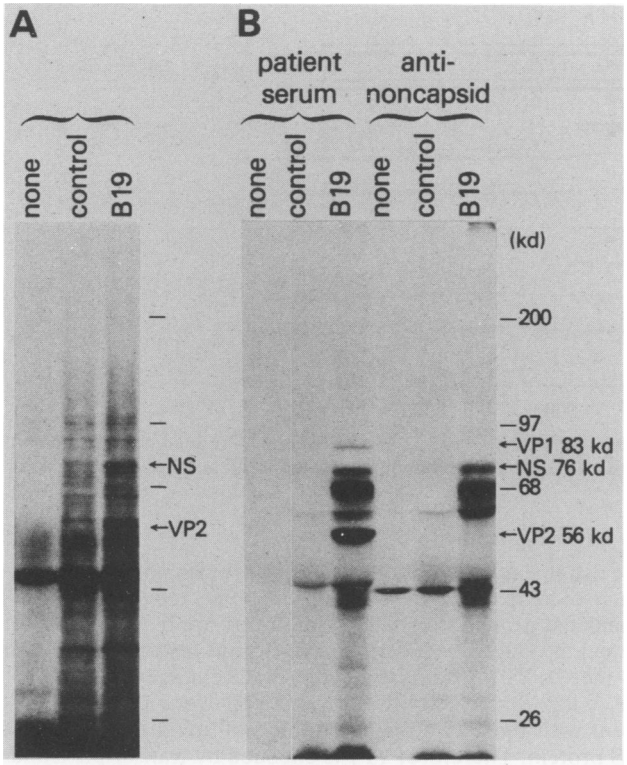

FIG. 1. In vitro translation of RNA from B19-infected erythroid bone marrow cells. RNA from cells infected in vitro and uninfected control cells was translated in vitro by rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Ten micrograms of total RNA was used for each reaction. A 1-μl sample of each reaction mixture (of a total of 50 μl) was directly analyzed by electrophoresis in 8% sodium dodecyl sulfate-polyacrylamide gels. B19-specific proteins were immunoprecipitated overnight from 49 μl of reaction mixtures with convalescent-phase serum or a rabbit antiserum to noncapsid protein. (A) Total translation products; (B) B19-specific translation products.

2.5 μl of diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.)-treated dH_2O. The reaction was allowed to proceed at 30°C for 1 h. One microliter of each reaction was subjected to electrophoresis in 8% sodium dodecyl sulfate-polyacrylamide gels (6). The remaining 49 μl of reaction mixture was immunoprecipitated with specific human or rabbit antiserum before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3, 13). The capsid protein VP1 of 83 kDa was visible as a faint band, and the major capsid species of VP2 was visible as a denser band at 56 kDa. Multiple B19 NS proteins were precipitated by using both the convalescent-phase human antiserum and a rabbit antiserum to the NS protein (3). At least two major species were observed: one of 76 kDa, similar to that seen in infected cells and on immediate analysis of the in vitro translation products (Fig. 1A), and a new, major band of 68 kDa. Intact infected cells also have multiple protein species that are precipitated with antinoncapsid antiserum (77, 52, and 34 kDa) (13). Some of the smaller polypeptides may represent degradation products within infected cells. However, the 68-kDa protein probably results with premature termination of translation of the NS gene RNA, since similar discrete bands of 76 and 68 kDa have been observed following in vitro translation of single synthetic RNA species derived from the left side of the B19 genome, and introduction of a mutation in the 3′-coding region of these same RNAs resulted in a single (truncated) translation product (data not shown). If the 68-kDa protein originated from the use of an internal AUG codon, this mutation would have been expected to produce two truncated proteins. A truncated version of noncapsid protein might also result from premature termination of transcription of RNA b in nonpermissive cells, although no such species has been detected in infected cultures of nonpermissive human bone marrow cells (13). However, other smaller protein species (Fig. 1B) observed with immunoprecipitation may have arisen from use of the internal AUG promoter, as are frequently obtained with the reticulocyte lysate system for in vitro translation.

In vitro translation after negative hybrid selection. Antisense oligomers of 18 nucleotides corresponding to selected regions of B19 RNA were synthesized on an automated solid-phase synthesizer (Applied Biosystems, Inc., Foster City, Calif.) by using standard phosphoramidite chemistry and were purified by high-pressure liquid chromatography (4). Oligomers (5′ to 3′) corresponded to the B19 genomic sequence (15) as follows: A, CTGTTAGTTAGCTCACAA, nt 389 to 406; B, AGCACAGTCTAGAACATT, nt 472 to 489; C, TGGGCTTCCGACAAATGA, nt 2092 to 2109; D, TAGGGGATTATCTAAAGA, nt 2585 to 2602; and E, AGTGCTGGCTTCTGCAGA, nt 3140 to 3157. Because of the absence of significant homology of the oligomers with other regions of the genome on computer search (Microgenie; Beckman Instruments, Inc., Palo Alto, Calif.), they were expected to bind to single sites. 18-mer A was used to remove the cap structure by cleavage within the short left-side leader sequences for all the B19 RNA species (Fig. 2). This oligomer should reduce translation of all B19 RNA species. 18-mer B was placed just 3′ to the translation initiation site of the left-side transcript (Fig. 2, b) and was anticipated to block translation from this transcript by cleavage without affecting translation from other RNA species. 18-mer C was selected to test for the presence of protein products from the abundant middle transcripts (Fig. 2, e and f); this oligomer also cleaves the right-side transcripts in the second exon to reduce translation efficiency by the removal of their capped structure; 18-mer C cleaves the left-side transcript b, which would result in 3′ truncation of an encoded protein. 18-mer D, just within the coding sequence of transcripts a and a′, should eliminate a protein product of these transcripts, while 18-mer E would abolish protein production by all right-side transcripts (a, a′, c, and d), except for a putative product of transcripts g and h.

For hybridization, 10 μg of sample RNA was lyophilized and suspended in 1 μl of diethyl pyrocarbonate-treated dH_2O, 1 μl of oligomer in diethyl pyrocarbonate-dH_2O, and 1 μl of 3× reaction buffer (300 mM KCl, 60 mM Tris hydrochloride [pH 7.5], 3 mM EDTA) in a 1.5-ml Eppendorf tube. After gentle mixing, the reaction mixture was heated to 65°C for 2 min to disrupt RNA secondary structure. RNase H (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was added as 1.5 μl of a solution of enzyme (25 U/ml) in reaction buffer (100 mM KCl, 20 mM Tris hydrochloride [pH 7.5], 4.5 mM $MgCl_2$, 3 mM dithiothreitol, 1 U of RNasin per μl, and 150 μg of bovine serum albumin per ml) and incubated at 37°C for 30 min. The RNase H-treated RNA samples were translated in vitro by rabbit reticulocyte lysate. The results of negative hybrid selection with these oligomers are shown in Fig. 3. Oligomer hybridization and RNase H digestion had no inhibitory effect on overall protein translation in vitro (panel A, total translation products). The

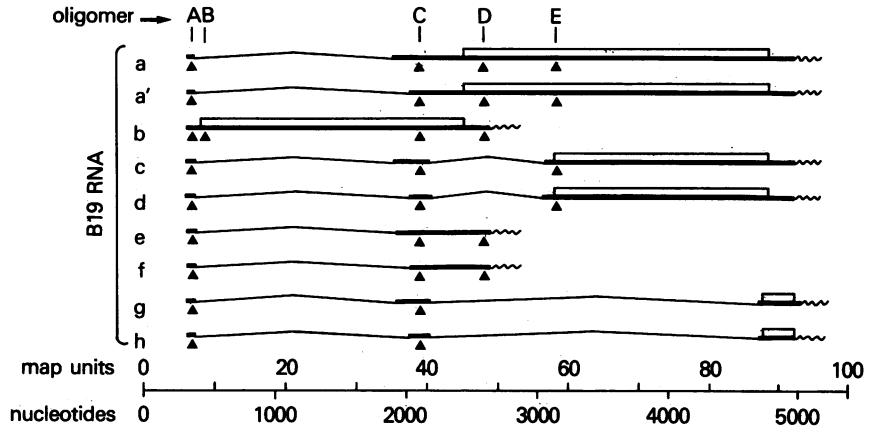

FIG. 2. B19 transcription map (10) showing location of sites of cleavage by synthetic oligomers. The B19 genome is conventionally divided into 100 map units, each of which is 54 nucleotides. B19 transcripts are labeled a to h. Protein-coding regions predicted based on sequence analysis are shown by open boxes.

oligomers did selectively inhibit specific B19 protein translation (panel B, immunoprecipitation with patient serum containing anti-B19 antibodies). As expected, removal of the cap structure by cleavage with 18-mer A resulted in slightly reduced translation of all B19 products, VP1, NS, and VP2, compared with either the control (no oligomer added, lane 2) or 18-mer D (note NS and VP2 intensities). 18-mer B markedly reduced the NS product. The 68-kDa band disappeared, and the 76-kDa band was decreased in intensity. This residual 76-kDa band may be artifactual, since a faint band of this size frequently appeared when RNA from uninfected cultures was translated; alternatively, we could not exclude the possibility that it represented a species of noncapsid protein synthesized from an aberrant mRNA or previously unmapped B19 parvovirus RNA. VP1 and VP2 were unaffected by 18-mer B, indicating that the NS protein was encoded by transcript b. 18-mer C treatment resulted in the disappearance of the 68-kDa NS species and the appearance of a new, 58-kDa band, the expected site of a 3'-truncated protein translated from transcript b. We could not detect products encoded by transcripts e and/or f. With 18-mer D, VP1 disappeared while VP2 remained unchanged, and with 18-mer E, both VP1 and VP2 were absent. Treatment with these two oligomers did not affect translation of NS protein. Therefore, VP1 is encoded by transcripts a and/or a', and VP2 is encoded by transcripts c and/or d.

Negative hybrid selection allowed direct correlation of B19 transcripts with protein products. B19 parvovirus conforms to the genomic organization of the *Parvoviridae*, with the left-side transcript encoding a nonstructural protein and the right-side transcripts encoding the capsid proteins. Negative hybrid selection is a convenient and efficient method for the analysis of protein products from overlapping transcripts.

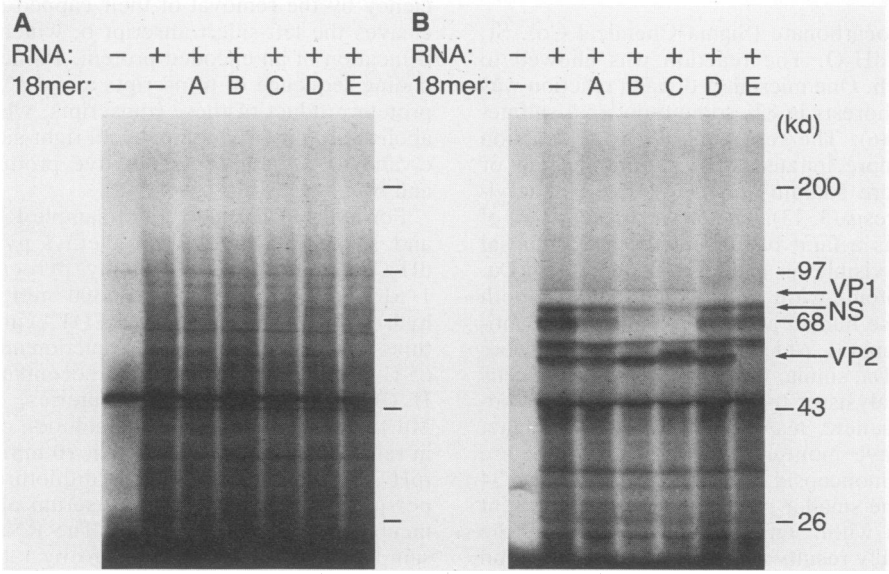

FIG. 3. Negative hybrid selection with antisense synthetic oligomers. In vitro translation products were analyzed after treatment of RNA with 18-mers A to E and RNase H. A 1-μl sample of the reaction mixture (total of 50 μl) was applied for determination of total reaction products, and 49 μl was immunoprecipitated with patient convalescent-phase serum for determination of B19 products. (A) Total translation products; (B) B19-specific products.

We thank Peter Tattersall for his kind gift of the pYT103 clone and Susan Cotmore for antibody to nonstructural protein.

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