PN ACM 555
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Progress Report - Israeli Group
Grant No.936-5544-G-00-7016-00

Densoviruses as Insecticides

July 1989

ABSTRACT

The Galleria mellonella densovirus (GmDNV) is a single-stranded DNA containing virus which infects the larval stages but not the adults. The small size (about 6 kb) makes this viral genome amenable for genetic analysis and manipulations, and the structural similarity to mammalian parvoviruses make it a convenient candidate for its development as an insect vector. To achieve this goal, we are studying the structure and expression of the virus in Galleria larvae. The sequence of about 40% of the genome was established, and the recently- constructed 22 chimera containing partially-overlapping viral DNA fragments in pUC 118 plasmid are being sequenced.B. Analysis of RNA transcripts

Northern blotting analysis revealed 5 polyadenylated, virus specific RNA species with approximate sizes of 1.8, 2.4, 3.5, 4.0 and 5.0 kilobases. S1 mapping studies of viral RNA are in progress. Hybridization of Northern blot of total viral RNA to probes representing different regions in the genome suggest that the transcriptional organization of the GmDNV genome is very similar to that of all other mammalian parvoviruses examined so far.

In vitro translation of RNA from GmDNV-infected larvae, suggests that the most abundant product, which co-migrated with the smallest, 46K viral polypeptide, may be coded by the most abundant 1.8 kb RNA species. In vitro translation of viral specific RNA, isolated by the hybrid selection method using viral DNA immobilized on nitrocellulose filters, yielded proteins with molecular weights of 30K, 46K, 50K, 60K, 66K and 68K and 90K. Experiments to determine the protein product(s) of each of the individual RNA transcripts are in progress.

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Materials and Methods.

Larvae, cells, virus and viral plasmids. Larvae of Galleria mellonella were grown on a food mixture containing Gerber's baby food (Mixed Cereal), honey and glycerol. Larvae were propagated in polypropylen boxes. For venting purposes, a stainless steel screen was welded into the box covers. The boxes were incubated at a constant 30°C temperature, with 50% humidity. The pupae were transferred to larger boxes without the addition of fresh food, and allowed to lay eggs on a multi-folded wax paper. To obtain synchronous larval cultures, eggs laid during a 24-48 h period were transferred to each box. To obtain an "all-year-round" culture, hatching times were controlled by incubating eggs at 4°C and seeding them in food boxes at pre-determined time intervals. Insect cell cultures were obtained from Dr. Dwight Lynn, from USDA in Beltsville, Maryland. Insect cells were grown at 30°C in Grace medium (GIBCO) supplemented with 10% fetal calf serum, and 0.3% each of lactalbumin and yeastolate (Difco). GmDNV was a gift from Dr. Peter Tijssen from Institut Armand Frappier, Montreal, Canada. The virus was grown in fourth instar larvae which were either injected or infected. Dead larvae were collected, ground, filtered through glass wool, and virus particles were purified according to published procedures. To construct a genetic clone, double-stranded viral DNA (see below) was cleaved with BamH1, and the resulting 5.4 kb fragment was ligated into the BamH1 site of pUC19.

Viral DNA and RNA. To obtain single stranded (ss) viral DNA, CsCl-purified virus particles were extracted with phenol-chloroform. Total RNA from infected larvae was prepared by grinding he larvae in 4M guanidine thiocyanate in a Polytron, filtering the extract through glass wool and subjecting the extract to isopycnic centrifugation in CsCl (1.4 g/cc, 12h at 30,000 rpm at 20°C). The RNA sediments at the bottom and was usually washed once with guanidine hypochloride, then phenol extracted. Poly (A) - containing RNA was selected by chromatographing the RNA through an oligo (dT)-cellulose column. Viral double-stranded (ds) DNA was obtained by two ways. It appeared as a distinct band in CsCl gradients during RNA preparation, and was extracted, and further purified by agarose gel electrophoresis. Alternatively, CsCl-purified virus was extracted at high ionic strength, yielding duplex viral DNA (see below).

Size analysis of viral proteins and RNA. To isolate viral proteins, CsCl-purified virus was suspended in 100 mM Tris-HCl pH 7, 10 mM EDTA buffer containing 5% SDS and 5M urea, and subjected to SDS-PAGE electrophoresis. Formaldehyde denaturing gel electrophoresis of RNA and blotting to nitrocellulose were done as described (Davis et al, 1986). The filter was baked in a vacuum oven (2 h, 80°C) and hybridized with cloned GmDNV DNA, labeled with ^{32}P by random prime-mediated incorporation to 1-3 X 10^9 cpm/μg, 10^7 cpm per 100 cm^2), washed and autoradiographed with Agfa Curix RPM2 X-ray film.

In vitro translation. Poly (A)-containing RNA (7 to 20 nanograms) from infected (48 h postinfection) and uninfected larvae was translated in a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (60 μCi), according to a published procedure (Davis et al, 1986) and the translation products were subjected to electrophoresis in an SDS- 8-30% polyacrylamide gradient gel.

Results

Cloning and sequencing of the GmDNV genome.

In the virion, GmDNV DNA (like all other parvoviruses) exists in a single-stranded form, which is converted to a duplex "replicating form" (RF DNA) during productive infection. RF DNA was isolated by two procedures. The first procedure was a simple phenol extraction of CsCl-purified virus preparation, In the presence of 0.1M NaCl. As reported below, the two strands of GmDNV DNA (viral and complementary, or + and - , respectively) are packaged into separate virus particles in an equimolar ratio. Phenol extraction readily yielded ds DNA, about 6 kb in size (see below). In the second procedure, viral RF DNA was obtained from injected larvae, as a side product of RNA preparation by the guanidine method, at the CsCl step. Using this procedure, viral DNA was obtained in ample quantities, but had to be further purified by agarose gel electrophoresis.

BamH1 cleavage of viral RF DNA conveniently yielded a 5.4 kb fragment which, as is shown below, contained the entire coding region of the virus. The BamH1-flanked fragment was ligated into pUC19, and a partial restriction map was constructed using, primarily, enzymes with sites represented in the polylinker of pUC19. Based on the restriction map, subclones representing the entire sequence

were subsequently constructed in M13. Using the dideoxy method and "Sequenase" (USB), about 2500 bases (from non-contiguous regions) were sequenced to date. The fragments at the termini of the viral DNA were given priority in sequencing. This was for two purposes. First, to find out whether this clone contained the entire coding region of GmDNV. Parvoviruses are characterized by terminal repeats on both ends. These repeats contain signals for DNA replication and packaging, but not coding sequences. Therefore, terminal repeats would indicate that the entire coding sequences are present in the clone. Indeed, computer analysis of the terminal regions (see previous report) showed a perfect inverted terminal repeat of at least 250 nucleotides. Therefore, the clone domains are the terminal repeats, tentatively suggesting (based, of course, on our knowledge from mammalian parvoviruses), that the coding sequences of the virus are entirely contained in the sequence.

The second purpose for the priority in sequencing the termini was a structural consideration. Surprisingly, the terminal repeat found, places GmDNV in that category of parvoviruses with identical termini (i.e., the human AAV and B19), as opposed to those containing, on both ends, palindromic structures, but containing unrelated sequences. There is, however, a major structural difference between that found in AAV and GmDNV. AAV DNA contains a <u>direct</u> repeat of 145 bases, and within it is a palindromic sequence of 125 residues that form a hairpin. The rest of the repeated sequence is short (20 residues), not involved in the hairpin structure, and called "inboard" sequence. In GmDNV, we are already finding an exceptionally long <u>inverted inboard repeat.</u> Hence, this is a unique structure within the parvoviruses, yet the general structural feature of terminal repeats, is maintained. Another unusual feature is the very high percentage of A and T stretches of 6 residues and over.

Computer analysis of the available sequence included homology searches against known sequences of mammalian parvoviruses, and also against that of Bombyx mori DNV (BmDNV), the only invertebrate parvovirus sequenced to date. Such searches revealed a complete lack of homology. Although this data is based on 50% of the sequence only, the lack of homology to BmDNV is surprising, since in mammalian parvoviruses, an extensive region of homology is found in the left ORF.

While sequencing restriction fragments yielded valuable information, the GmDNV genome has few unique restriction sites which makes it difficult to isolate fragments with manageable sizes.

Therefore, we have cloned the 5.4 kbp fragment into pUC 119 plasmid, and created a series of nested deletions using Exonuclease III, followed by S1 nuclease digestion and blunt-end ligation. Single-stranded DNA was isolated from each of the deletion mutants by infecting cells with a helper KO7 phage. Sequencing of these fragments is now in progress.

Composition of GmDNV density classes.

Extracts of GmDNV-infected G. mellonella yielded, in aqueous CsCl gradients virions of 3 density classes, i.e., at 1.32. 1.40 and 1.42 g/ml (Fig. 1). We have confirmed previous reports that density classes 1.42 and 1.4 are infectious , and that the lighter, 1.32 band ("top component") contains non-infectious virus (Kelly et al, 1977; Tijssen and Kurstak, 1981; Chao et al., 1986; Mari and Bonami, 1988.). Alkaline agarose gel electrophoresis of the 3 density classes (Fig. 1), confirmed that the 1.32 band contains particles devoid of DNA.

The proteins of the 3 density classes were analyzed by SDS-PAGE electrophoresis. The results, presented in Fig. 2, show that 4 polypeptides were common to all 3 bands. Their molecular weights were in good agreement with those determined previously, namely 46K, 53K, 62K and 94K (Tissen et al, 1982). The 1.32 band contained two additional polypeptides with molecular weights of 66,000 and 68,000. The relative abundance of the viral proteins in all 3 density classes varied extensively from one preparation to another. Consistently, however, the 94K polypeptide in the 1.32 band was by far the least abundant, and sometimes almost invisible in Coomassie Blue staining.

The most surprising finding, however, was the detection of extremely high quantities of the 46K and 48K proteins seen in the "empty" virions (1.32 g/cc), also at the top fractions of CsCl gradients. These proteins are clearly not associated with viral particles, but are of viral origin since they appear in the in vitro translation products of injected larva-RNA, and they are also produced in in vitro translation of hybrid-selected viral RNA (see below). As shown in Fig. 4, the abundance of these proteins in these top fractions exceeds by far that of all other viral proteins in the particle-associated fractions. We are currently carrying out tryptic digests of these two new components, in order to determine their relationship to the other structural proteins.

Structure of GmDNV DNA.

There has been one earlier report that extraction of DNA from virus particles in the presence of high salt resulted in ds DNA, while extraction in low salt yielded ss DNA. We repeated this finding using gel electrophoresis and blotting techniques, which were not available at that time. Indeed we found that DNA extracted in the presence of 0.1 M NaCl migrated in neutral agarose gel as a 6 kbp, while DNA extracted in the absence of NaCl migrated as a 6 kb fragment. Thus, GmDNV seems to package viral and complementary DNA strands in separate virions in a fairly close molar ratio. The only other parvoviruses known to do that are the human AAV and B19, and none of the ones in between in the evolutionary scale.

In vitro translation of RNA from infected larvae.

The sequence of a 5.4 kbp DNA clone of GmDNV is currently being determined. Sequences of the termini of the clone, already available, show that they contain inverted repeats 265 nucleotides inside from the BamHI site. The genomes of all mammalian parvoviruses contain terminal repeats which form hairpin structures, and which flank the viral coding region. The termini lack sizable open reading frames (ORF's) (the longest one is 23 amino acids from the first methionine), thus the 5.4 kbp clone contains the entire coding region of GmDNV (P. Tijssen, manuscript in preparation; , M. Huleihel, T. Feldman and J. Tal, unpublished results). The GmDNV coding region is, therefore, approximately 5,000 bases, and is clearly insufficient to code for the 6 or even 4 viral proteins found. To determine which of them were primary translation products, RNA from infected larvae was translated in vitro in a rabbit reticulocyte lysate, and the translation products were co-electrophoresed with proteins extracted from CsCl-purified virus. The most abundant in vitro translation product co-migrated with the smallest viral protein, p46 (Fig. 2). The 46K polypeptide is not, however, the most abundant protein in purified virions. The ratios of the 4 viral proteins varied widely between virus preparations, suggesting that its abundance in virions is subjected to translational or post-translational control.

We have also purified viral RNA by hybridizing it to immobilized GmDNV DNA and eluting it from the filters. This hybrid-selected RNA was translated in vitro, yielding polypeptides with the

molecular weights of 30K, 46K, 50K, 60K, 66K and 68K and 90K, approximately (Fig. 6). This large number of polypeptides is quite surprising. To conclusively determine the number and nature of the viral proteins we are currently using anti-GmDNV antiserum (raised in rabbits in our laboratory) to immunoprecipitate the in vitro translation products. In addition, we are doing tryptic digest analysis to all the products.

Size analysis of viral mRNA.

The viral transcripts synthesized in vivo were analyzed by northern blotting. Larvae were injected with virus, and 48 h later RNA was isolated by the guanidine method, as outlined in the Materials and Methods section. The RNA was then fractionated to poly $(A)^+$ and poly $(A)^-$ RNA by chromatography through a column containing oligo-dT cellulose. Both RNA fractions were electrophoresed in a formaldehyde agarose gel, blotted onto a nitrocellulose filter paper and hybridized with ^{32}P-labeled, cloned viral DNA. To ensure that the RNA was not degraded, an additional lane in which poly $(A)^-$RNA was run was cut out from the gel, washed with water and stained with ethidium bromide. Only RNA which yielded discrete ribosomal RNA bands and lacked trails of degraded material was further blotted and processed. This analysis revealed 5 polyadenylated, virus specific RNA species with approximate sizes of 1.8, 2.4, 3.5, 4.0 and 5.0 kilobases (Fig. 3). Their ratios, as determined by densitometry, were roughly 4 : 2.3 : 1 : 1 : 1, respectively. We could not detect non-adenylated transcripts, even with large amounts of poly $(A)^-$ RNA.

Initial mapping of the transcripts is being done by hybridizing Northern blots of viral RNA to labeled probes from defined regions in the genome. This is a preliminary approach, while S1 mapping is underway, intended to provide us with an idea of the location of the transcripts. The data so far is consistent with that of all other parvoviruses analyzed to date. Fig. 5 (although poorly-reproduced in the enclosed photocopies), shows that the right-hand fragments hybridized to all 5 transcripts (lane 2), Also, the intensity of the 1.8 kb transcript is unchanged with both right-hand probesright hand probes (lanes 2 and 3) suggesting that all transcripts terminate at the right hand side, and possibly a common polyadenylation site as in mammalian parvoviruses. The 1.8 transcript is missing when the left-hand

HincII fragment is used, suggesting that the 1.8 kb transcript, which codes for the viral structural 46K protein, initiates further downstream, again as is the case with mammalian parvoviruses.

DISCUSSION

We characterized, for the first time, the transcripts of densonucleosis virus. Comparison of the viral proteins to those synthesized in vitro, shows that the smallest and most abundant viral protein, the 46K, co-migrates with the smallest structural viral protein, and therefore it is, most probably, a primary translation product. The 53K protein also co-migrates with an in vitro translation product which is not found in the control of uninfected larva RNA. Interestingly, two polypeptides among the translation products seem to correspond to the 66K and 68K virus-extracted proteins, which are only present in the empty capsids component. The nature of these polypeptides is not clear, but their absence from the other two components may suggest that they are non-structural (NS) viral proteins.

Five size classes of polyadenylated RNA were found, with sizes of 1.8, 2.4, 3.5, 4.0 and 5.0 kilobases. All five mRNAs were polyadenylated, and none were found in the poly $(A)^-$ fraction of the oligo (dT)-cellulose column. In this respect, GmDNV is similar to the autonomously-replicating parvoviruses, and not to the adeno-associated viruses which generate both polyadenylated and non-adenylated messages.

As we learn more about densonucleosis viruses, similarities to mammalian parvoviruses are becoming obvious. From the structural aspect, both contain 3 density classes with about the same buoyant density as found in mammalian parvoviruses. Moreover, the lighter density class is composed of "empty" virus particles, i.e., particles which contain the protein capsids only, and no DNA. Another similarity is in the number and size distribution of the viral proteins, and in the variable ratios of these proteins between virus preparations. The palindromic sequence repeat at the genome termini is also maintained, although in GmDNV it is unusually long and inverted (Tijssen, unpublished results; Huleihel, Feldman and Tal, unpublished results). We show in this work that this similarity can be extended to viral gene expression as well. With respect to the viral transcription, two interesting points

are worth emphasizing: one of the RNA transcripts is a genome length one, and the smallest transcript is also the most abundant one. Both these features are strikingly similar to mammalian parvoviruses. Furthermore, in mammalian parvoviruses, the most abundant transcript codes for the viral structural proteins, and it is tempting to speculate that this is also the case in GmDNV, since the most abundant, 1.8kb viral mRNA species, is only slightly larger than the template required for the synthesis of the major in vitro synthesized 46K polypeptide.

Another similarity that can be drawn between GmDNV and mammalian parvoviruses is the size of viral proteins, which together exceed by far the coding capacity of the viral genome. Mammalian parvoviruses contain partially overlapping transcription units (Pintel et al., 1983), and further expand their coding capability for the production of different proteins by alternate splicing of the mRNA (Morgan and Ward, 1986; Jongeneel et al., 1986; Becerra et al., 1988; Trempe and Carter, 1988; In GmDNV, all four structural proteins have extensive sequence homologies (Tijssen and Kurstak, 1986), thus arguing against separate coding regions for each of them. Further research is required to determine if the mechanisms found in mammalian parvoviruses are also employed in the expression of GmDNV.

LITERATURE CITED

Becerra, S. P., Koczot, F., Fabisch, P., and Rose, J. A. 1988. Synthesis of adeno-associated virus structural proteins requires both alternative messenger RNA splicing and alternate initiations from a single transcript. J. Virol. 62, 2745-2754.

Chao, Y.-C., Young III, S. Y., Kim, K.S., and Scott, H. A. 1985. A newly isolated densonucleosis virus from Pseudoplusia includens (Lepidoptera: Noctuidae). J. Invertebrate Pathol. 46, 70-82.

Davis, L. G., Dibner M. D., and Battey J. F. 1986. Basic Methods in Molecular Biology. Elsevier Science Publishing Co., New York.

Kelly, D.C., Barwise, A.H. and Walker, I.O. 1977. DNA contained by two densonucleosis viruses. J. Virol. 21, 396-407.

Mari, J., and Bonami, J.-R. 1988. PC 84, a parvo-like virus from the crab Carcinus mediterraneus: pathological aspects, ultrastructure of the agent, and first biochemical characterization. J. Invertebrate Pathol. 51, 145-156.

Morgan, W. R., and Ward, D. 1986. Three splicing patterns are used to excise the small intron common to all minute virus of mice RNAs. J. Virol. 60, 1170-1174.

Pintel, D., Dadachanji, D., Astell, C.R., and Ward D. C. 1983. The genome of minute virus of mice, an autonomous parvovirus, encodes two overlapping transcription units. Nucleic Acids Res. 11, 1019-1038.

Tijssen, P. and Kurstak E. 1981. Biochemical, biophysical and biological properties of densonucleosis virus (parvovirus). III. Common sequences and structural proteins. J. Virol. 37, 17-23, 1981

Trempe, J. P., and Carter, B. J. 1988. Alternate messenger RNA splicing is required for synthesis of adeno-associated virus VP1 capsid protein. J. Virol. 62, 3356-3363.

Legends to Figures

Fig. 1. Density classes of GmDNV and their DNA content. Left, Density classes of GmDNV obtained in equilibrium centrifugation in CsCl. Right, A sample from each density class was subjected to alkaline agarose gel electrophoresis. The gels were then neutralized and stained with ethidium bromide.

Fig. 2. In vitro translation of poly (A)-containing RNA from GmDNV-injected larvae. Viral RNA from infected and uninfected larvae, was prepared as described in the Materials and Methods section and translated in a reticulocyte lysate system. The translation products were compared by gel electrophoresis to viral proteins from each of the 3 density classes. M, Molecular weight markers; A and B, coomassie blue staining of translation in the absence of externally-added mRNA, and with 20 nanograms of poly (A)-containing RNA, respectively; C and D, corresponding autoradiograms of A and B; E,F,G, viral proteins from density classes 1.32. 1.42 and 1.44, respectively.

Fig. 3. Northern analysis of GmDNV RNA. Viral RNA was extracted from infected larvae, and electrophoresed in a formamide gel as described in Materials and Methods. A, 0.5 μg poly $(A)^+$ RNA; B, 16 μg poly (A) RNA. The relative amounts of the RNA size classes were calculated from the tracing shown, performed in an Hoeffer GS 350 densitometer.

Fig. 4. Detection of a 66K-68K protein component. GmDNV was purified from injected larvae and purified on a CsCl gradient. Fractions were collected and a sample of each was analyzed by SDS-PAGE electrophoresis. The positions of the 1.32, 1.4 and 1.42 density classes are marked.

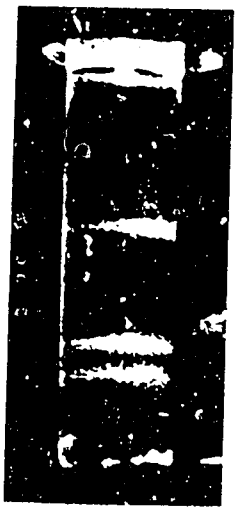
Fig. 5. In vitro translation of hybrid-selected RNA from GmDNV-injected larvae.

Fig. 6. Northern analysis of GmDNV RNA. Northern blots were hybridized with ^{32}P-labeled DNA probes derived from the left and right parts of a cloned GmDNV genome. Lane 1, genomic clone; lane 2, $EcoRI_{(4.0)}$ -$EcoRI_{(5.5)}$; lane 3, $EcoRI_{(3.8)}$-$EcoRI_{(4.0)}$; lane 4, $HincII_{(0)}$-$HincII_{(0.5)}$.

PUBLICATIONS

1. Gross, O., Weinberg, D., Tijssen, P. and Tal, J. (1989). Expression of Densonucleosis virus GmDNV in Galleria mellonella larvae: size analysis and in vitro translation of viral transcription products. J. Invertebrate Pathol., Accepted for publication.

2. Gross, O., and Tal, J. (1989). Strand orientation and mapping of Galleria mellonella densovirus RNAs. In preparation.



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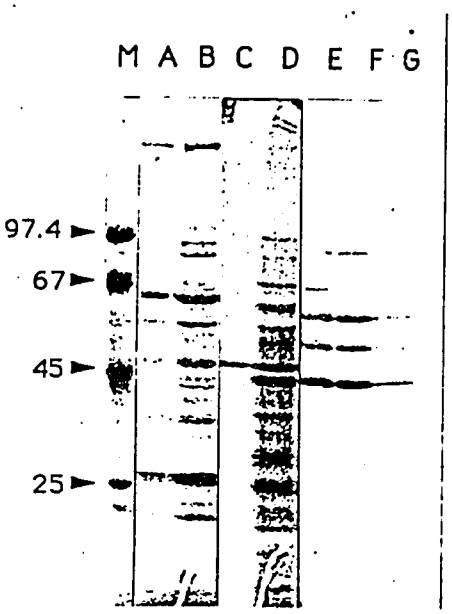
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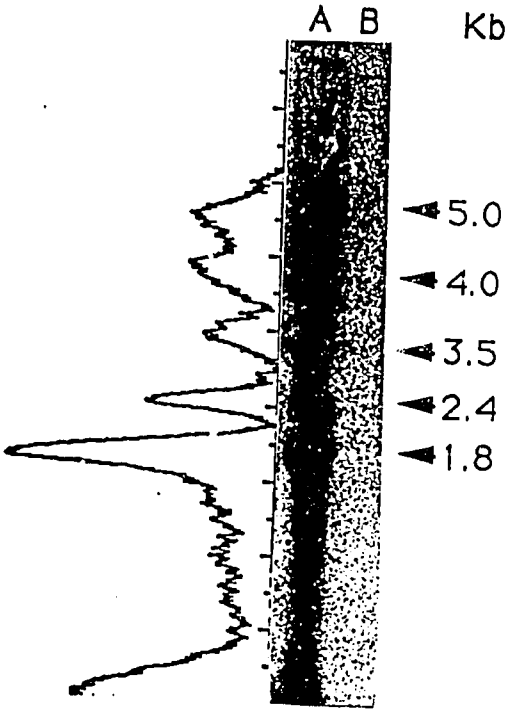
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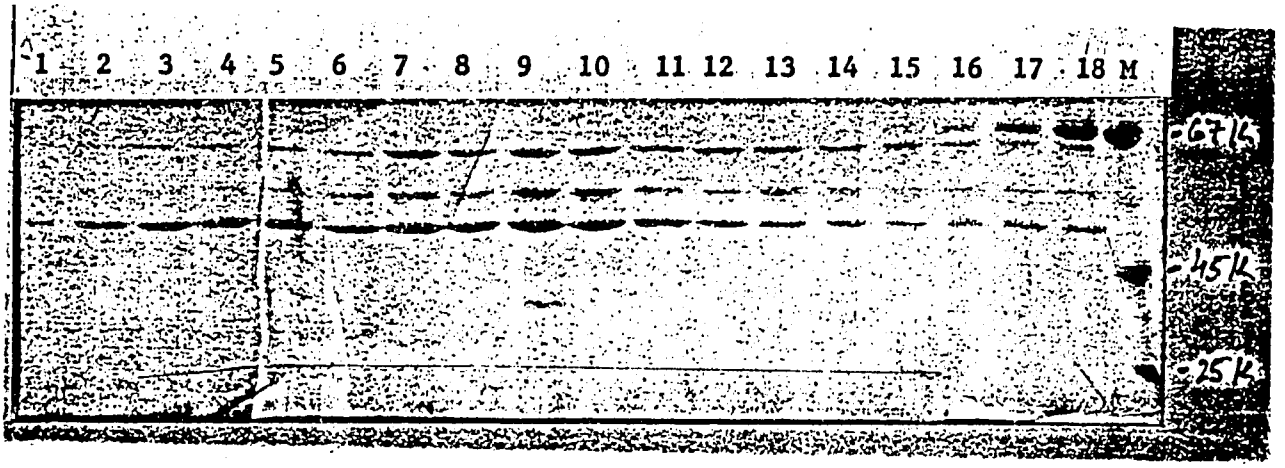
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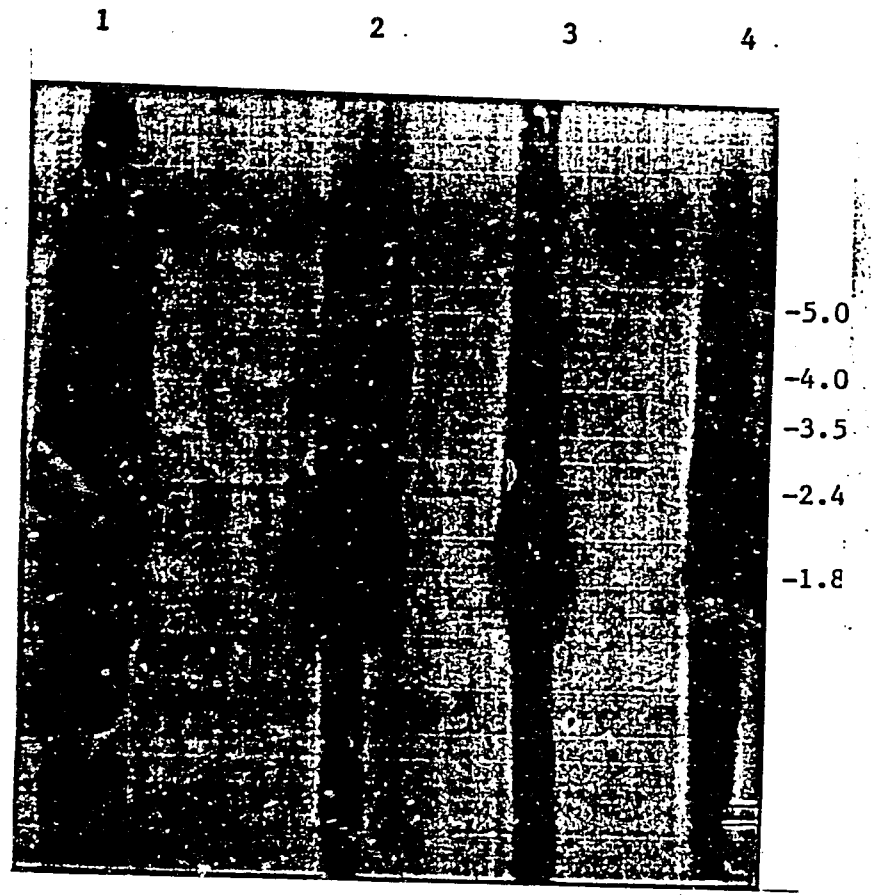
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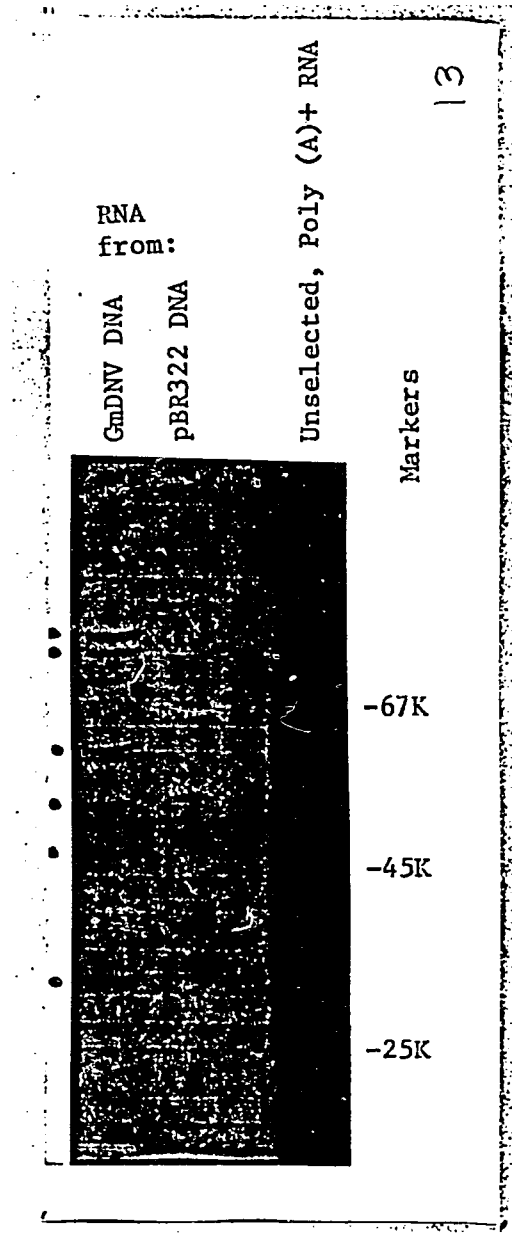
RNA
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pBR322 DNA
Unselected, Poly (A)+ RNA
Markers
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-45K
-25K

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