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Transcriptional Mapping of Rabies Virus In Vivo

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Synthesis of the proteins of rabies virus was studied in hamster cells infected with UV-irradiated virus. The UV target size of genes L, N, M_1, and M_2 was measured during primary transcription. Except for N, the target size of the remaining genes was considerably larger than that of their physical sizes. The data fit the hypothesis that four genes occupy a single transcriptional unit and that transcription of rabies virus proceeds in the order N, M_1, M_2, and L.

It has been demonstrated that rabies virus is a negative-strand virus, like other rhabdoviruses, because its RNA is not infectious (15), and RNA polymerase activity in purified virions has been demonstrated in vivo (5) and in vitro (8a, 10). Rabies is insensitive to actinomycin D and cytosine arabinoside at doses compatible with host cell survival for 24 h (9). RNA synthesis of this virus in vivo resembles that of vesicular stomatitis virus (VSV), although it proceeds at a lower rate and with a lower yield (8).

The molecular weight of rabies virus RNA is approximately 4.6×10^6 (14, 15). The RNA molecule is associated with a phosphorylated nucleoprotein (N) in the virion, forming a nucleocapsid core. The latter is surrounded by a viral envelope containing two nonglycosylated membrane proteins (M_1 and M_2) and one glycoprotein (G). A high-molecular-weight polypeptide, approximately 1.9×10^5, designated polypeptide L, has been detected in both virions and infected cells (10, 12). It is probably associated with the transcriptase activity.

By using the method pioneered by Sauerbier (13), the sequential nature of primary transcription in VSV in vivo and in vitro has been demonstrated by analyzing the effects of UV radiation of VS virions on subsequent RNA and protein synthesis. An UV lesion in the genome impairs the transcription of those genes located beyond the lesion, towards the 5′ end of the nucleotide chain (1-3). The target size of each gene is equal to the cumulative physical sizes in the following increasing order: N, NS, M, G, and L. These results were unambiguously obtained at the onset of the viral replicative cycle in vivo. Late after infection, complexities arise, probably due to the preferential replication of undamaged genomes.

The present report utilizes the same reasoning to investigate the possibility that rabies virus transcription is also sequential.

MATERIALS AND METHODS

Viruses and cells. The Orsay stock of rabies virus is a clone of the CVS strain titrating at 1.5×10^7 PFU/ml. Viral multiplication was in BHK-21 cells. In addition, a hamster cell line (CER, kindly provided by T. J. Wiktor) was used in which rabies forms plaques under agarose (9). Protein synthesis resulting from primary viral transcription was studied with CER cells, because the inhibition of cellular protein synthesis was more satisfactorily obtainable with this line than with BHK-21.

Preparation of concentrated virus. Two bottles of BHK 21 cells, containing 3×10^7 cells per bottle, were infected with the Orsay stock of rabies virus at a multiplicity of infection (MOI) of 0.1 PFU/cell. After a 30-min adsorption at room temperature, infected cells were incubated in Eagle medium supplemented with 0.1% (wt/vol) bovine serum albumin (0.1% BSA-MEM, 80 ml per bottle in a 5% CO_2 atmosphere). After 3 days at 37°C, the supernatant was harvested, clarified by centrifugation at 1,500 rpm for 15 min, and finally centrifuged at 30,000 rpm for 90 min in a Beckman 42 rotor. The resulting pellets were suspended in 2 ml of 0.1% BSA-MEM. Titers before and after concentration were 2×10^7 to 4×10^7 and 10^9 to 2×10^9 PFU/ml, respectively.

Irradiation of viruses. Concentrated virus was irradiated in glass wells (150 μl of suspension per well) with an UV germicidal lamp. Radiation doses were determined with a Latarget dosimeter, and virus particles were used immediately for infection with CER cells. The inactivation curve was determined by sampling portions.

Preparation of labeled virus. One bottle of confluent BHK-21 cells (3×10^7 cells) was infected with the Orsay stock of rabies virus at an MOI of 0.1 PFU/cell. An adsorption period of 30 min at room temperature was followed by an incubation of infected cells in 80 ml of 0.1% BSA-MEM for 2 days at 37°C in 5% CO_2. The medium was then replaced with 80 ml of 0.1% BSA-MEM containing 1 mCi of [^{35}S]methionine (CEA, Saclay, France), and the incubation was continued for 1 more day. The supernatant was then harvested, and virus particles were concentrated as described above, except that the pellet of virus particles was resuspended in 1 ml of 0.1% BSA-MEM. The

concentrated suspension was then centrifuged through a linear 15 to 30% (wt/wt) sucrose gradient in a Beckman SW 27 rotor at 25,000 rpm for 70 min, after which the viral band was clearly visible at three-quarters from the top of the tube. Thirteen fractions of 1.3 ml each were then collected from the bottom of the tube. Virus-enriched fractions 3 and 4 were then mixed with cellular extracts from an uninfected petri dish of unlabeled BHK-21 cells and treated as the other extracts (see below).

Inhibition of cellular protein synthesis. Two inhibitory procedures were employed. Cells were either UV irradiated immediately before infection with 1,000 or 1,500 ergs/mm^2 or were treated 30 min before labeling with hypertonic amino acid-free medium (Earle salt solution supplemented with Earle vitamins, 10% fetal calf serum, and an excess of NaCl [600 mOsM] as described by Madore and England [12]).

Infection and labeling of cells. Growth medium was removed from confluent monolayers of CER cells (3×10^6 cells) in 60-mm tissue culture dishes. Cells were then infected with 0.1 ml of the concentrated virus suspension (pre-irradiation titer 10^9 to 2×10^9 PFU/ml) or were sham infected with the same volume of 0.1% BSA-MEM. Adsorption was allowed to proceed for 30 min at room temperature, and cells were then incubated at 37°C for 3 h in 3 ml of BSA-MEM per dish in 5% CO_2. The cells were then pretreated with iso- or hypertonic amino acid-free medium (depending on the method of protein synthesis inhibition) for 30 min. Cells were labeled from 3.5 to 5 h after infection in iso- or hypertonic amino acid-free medium containing 50 μCi of [^{35}S]methionine at a final specific radioactivity of 50 to 100 Ci/mol.

Preparation of cell extracts. Iso- or hypertonically labeled cells were twice washed with ice-cold TD buffer (0.15 M NaCl-0.01 M Tris-hydrochloride, pH 7.4) and were scraped from the tissue culture dish in 2 ml of the same ice-cold buffer. A 10-ml quantity of ethanol was added to the cell suspension. The mixture was kept overnight at −20°C and centrifuged at 10,000 rpm in a Sorval swinging-bucket rotor for 15 min. After drying the pellets, they were dissolved in 150 μl of protein dissociation buffer (62.5 M Tris-hydrochloride, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue). Samples were kept at room temperature for 1 h and were then boiled for 1 min and stored at −70°C until use. Protein concentrations were determined as described by Bramhall et al. (7).

Polyacrylamide gel electrophoresis. Samples (50 μl) of cell lysates and virus samples were subjected to electrophoresis in discontinuous 10% Laemmli gel slabs containing sodium dodecyl sulfate (11). Electrophoresis was for 4 h at 80 V. After the run, gels were fixed in methanol-acetic acid-water (3/1/6, vol/vol/vol), were dehydrated in dimethylsulfoxide, imbibed with 20% 2,5-diphenyloxazole in dimethylsulfoxide, dried, and exposed to RP Royal "X-Omat" film at −70°C, as described by Bonner and Laskey (6).

Scanning of autoradiograms. Because each viral protein was present in infected cells at different concentrations, various exposures of the gel were made to record the viral bands. Autoradiograms were scanned with a Kipp and Zonen DD_2 densitometer coupled to a Hewlett Packard 3380A integrator-calculator with a low-slope sensitivity (0.03 mV/min). Intensities of the viral bands were recorded for each UV dose and corrected for background in the same region of autoradiograms from uninfected cells and also for protein concentration in the extract.

RESULTS

Viral protein synthesis in CER cells. Viral proteins are normally undetectable in infected cells, because rabies virus has no appreciable effects on cell protein synthesis. The latter can, however, be selectively decreased by treatment with a high-salt medium, as shown by Madore and England (12), or by irradiation with UV light. Under these conditions, bands comigrating with labeled proteins extracted from purified rabies virions were clearly present in cytoplasmic extracts from infected cells. As expected, they were absent in noninfected cells (Fig. 1, panels O, V, and C). Because early protein synthesis, i.e., synthesis corresponding to primary transcription, is consistently less abundant than late protein synthesis, high tonicity (600 mOsM), or strong UV irradiation (1,000 or 1,500 ergs/mm^2) had to be used to detect viral bands. When cells were pretreated with high tonicity, viral bands N, M_1, and M_2 could be detected (Fig. 1); osmolarities greater than 600 mOsM depressed viral protein synthesis as well (data not shown). UV irradiation of cells before infection also depressed cellular protein synthesis, with large polypeptides being more affected than small species. Under these conditions, viral proteins L, N, and M_1 could be detected in infected cells (Fig. 2). Neither of these methods led to the detection of G protein.

Two other critical factors were the MOI and the period of labeling. Considerable amplification of viral synthesis occurred after 5.5 h (Fig. 3), indicating that the replication of infecting viral genomes had already taken place by that time. Because our purpose was to study the transcription of irradiated virus particles, it was necessary to work during a period when the multiplication of undamaged genomes was only a minor phenomenon. In the experiments reported below, cells were labeled between 3.5 and 5 h postinfection.

As expected, protein synthesis corresponding to primary transcription was proportional to the MOI. At an MOI less than 10 PFU/cell, no viral bands could be detected in infected cells during the first 5 h of the viral cycle. Between 10 and 50 PFU/cell, only protein N could be clearly detected and the visualization of proteins L, M_1, and M_2 required at least 50 PFU/cell (compare Fig 3a [MOI = 10] with Fig. 1 and 2, panel 0

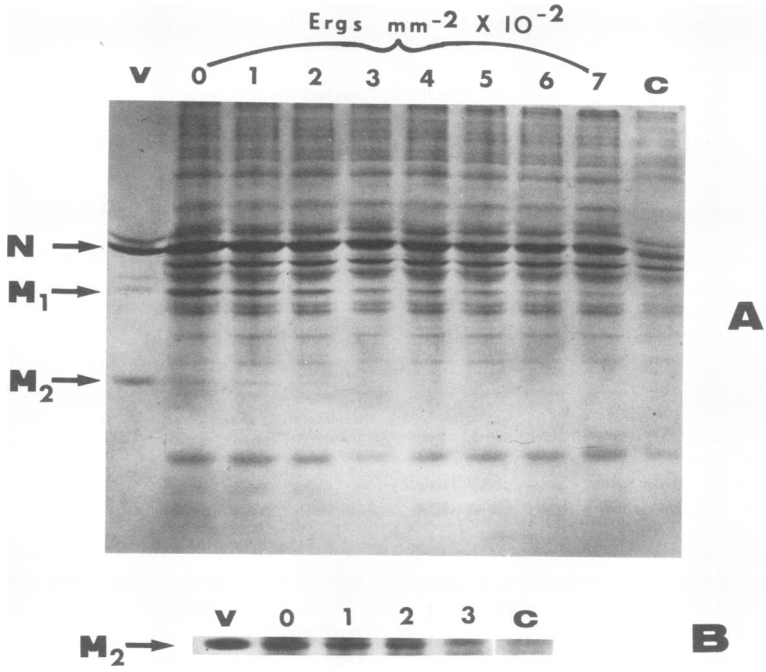

FIG. 1. *Viral protein synthesis after UV irradiation of virus particles. Cellular protein synthesis was depressed by high-salt treatment. Autoradiographs of a 10% discontinuous sodium dodecyl sulfate-polyacrylamide gel, after fluorographic treatment. CER cells were infected with UV-irradiated and non-irradiated rabies virus at a MOI equivalent to 50 PFU of unirradiated virus per cell. Cells were labeled from 3.5 to 5 h after infection with [^{35}S]methionine (100 μCi/ml). The position of proteins N, M_1, and M_2 is indicated (L and G are not detectable with this technique). (A) 4-h exposure of the autoradiogram. (B) 15-h exposure of the same gel (note the decrease of protein M_2). V, Proteins from purified labeled virus; 0, proteins from cell infected with unirradiated virus; 1–7, proteins from cells infected with virus irradiated with 100 to 700 ergs of UV light per mm^2; C, control (proteins from uninfected cells). In longer exposures of the same gel, proteins L and G from purified labeled virion can be seen. L is found as a sharp band at the origin of the gel, whereas G yields a diffuse band before N.*

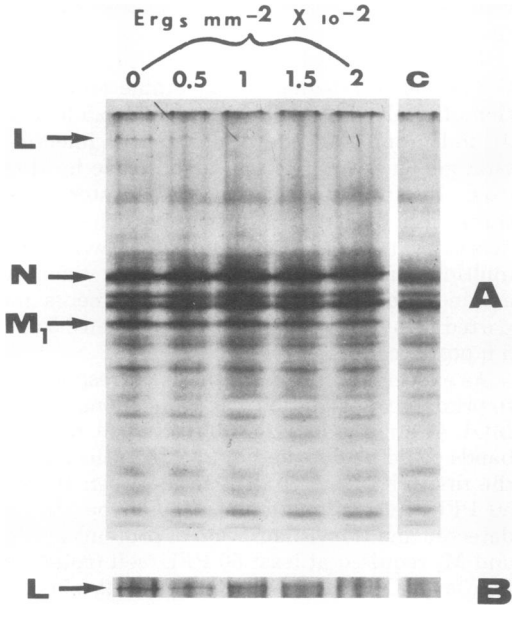

FIG. 2. *Autoradiograms of viral protein synthesis after UV irradiation of viral particles. Cellular protein synthesis was depressed by irradiating cells with 1,500 ergs of UV light per mm^2 before infection. (A) 2-h exposure of the gel; (B) 4-h exposure. See Fig. 1 and text for experimental details and for explanation of symbols.*

[MOI = 50]). All experiments were thus performed at an MOI of 50 to 100 PFU/cell.

Differential inactivation of viral polypeptide synthesis. Irradiation of rabies virus with increasing doses of UV light led to a decrease of infectivity and of the synthesis of proteins L, N, M_1, and M_2 with apparently single-hit kinetics (Fig. 4). The D_0, corresponding to one hit per genome or 37% survival of the population, was 100 ergs/mm^2 (Fig. 4A). The inactivation kinetics of the various viral proteins were not identical. Because target size for infectivity is equal to 4.6×10^6 daltons, the target sizes of genes N, M_1, M_2, and L can be calculated from their inactivation curves. It can be seen

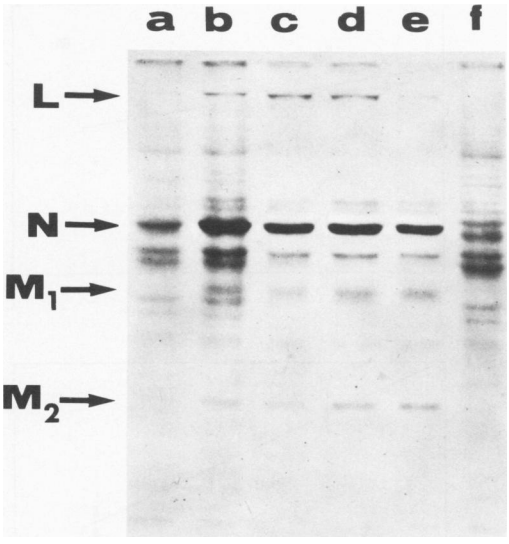

FIG. 3. *Autoradiograms of rabies protein synthesis in infected CER cells. Cellular protein synthesis was depressed by irradiating cells with 1,500 ergs of UV light per mm² before infection. Cells were infected with the CVS strain of rabies virus at a MOI of 10 PFU/cell and incubated at 37°C. They were labeled with [35S]methionine during 1.5 h at various times after infection in isotonic amino acid-free medium as explained in the text. The position of viral proteins L, N, M1, and M2 are indicated. a, Cells labeled 3.5 h postinfection with 50 μCi of [35S]methionine per ml; b, cells labeled 5.5 h postinfection with 50 μCi of [35S]methionine per ml; c, cells labeled 9.5 h postinfection with 25 μCi of [35S]methionine per ml; d, cells labeled 16.5 h postinfection with 12.5 μCi of [35S]-methionine; e, cells labeled 22.5 h postinfection with 12.5 μCi of [35S]methionine per ml; f, proteins from uninfected cells after 1.5 h of labeling with 50 μCi of [35S]methionine per ml.*

TABLE 1. *Comparison of target size and physical size of rabies proteins N, M1, M2, and L*

Protein	Protein mol wt[a]	Gene size (daltons)	Target size[b] (daltons)	Standard error target size[c]
N	60,000	540,000	506,000	±280,000
M1	45,000	405,000	1,370,000	±220,000
M2	20,000	180,000	1,690,000	±320,000
L	190,000	1,710,000	4,600,000	±1,100,000

[a] According to Sokol (16) and to Delagneau (unpublished data).

[b] Target size of the gene was calculated from the slope of the inactivation curve.

[c] Confidence limits of the target sizes were calculated from the standard errors on the slopes of the inactivation curves.

(Table 1) that gene N is the only one in which target size is equal to physical gene size. This suggests that either gene N is transcribed independently of the rest of the genome or that it is located at the beginning of the transcriptional unit. Although gene M_1 is physically smaller than N, its target size was three times greater than that of N. Similarly, protein M_2 is coded by a gene smaller than N and M_1, but its target size was about half the size of the genome. Finally, the inactivation kinetics of protein L were approximately those of infectivity; its target size is thus equal to the entire genome. The independent transcription of genes N, M_1, M_2, and L is not consistent with the present data. Rather, the data suggest that the four genes are sequentially transcribed in the order 3′-N-M_1-M_2-L-5′.

Strictly speaking, transcriptional mapping data give an order of transcription, not a physical order of the genes. Nevertheless, because it is probable that one reflects the other, the above data were used to derive a physical map of the rabies genome. On this hypothetical map, the target size of each gene is shown left to this point (Fig. 5). It can be seen that gene N is located close to the 3′ extremity and that gene L is at the 5′ end. Genes do not overlap and are apparently separated by three gaps. The larger gap is located between proteins M_2 and L and is sufficient to accommodate gene G (molecular weight, 738,000). But statistical analysis of the results does not enable us to eliminate the possibility that gene G is located between N and M_1.

DISCUSSION

The present results are consistent with the hypothesis of a sequential transcription of the rabies genome. This observation is similar to that obtained with VSV and suggests that the molecular biology of these two viruses is similar. In addition, the gene order of rabies is similar to that of VSV and would be even more closely related if rabies gene G was located after M_2.

Rabies genes are apparently separated by gaps, at least one of which is sufficiently large to code for a polypeptide as large as protein G. It should be noted that the rabies genome is larger than that of VSV (4.6×10^6 versus 3.8×10^6 daltons). Because the sizes of the five known rabies and VSV proteins are similar, either rabies codes for additional, as yet unidentified, proteins, or there are large, noncoding regions in its genome. A third possibility involves the overestimation of the size of the rabies genome, suggested by the observation of Bishop et al. (4) that RNA extracted from rabies and VSV virions comigrates in polyacrylamide gel electrophoresis.

Although we could not detect the presence of G protein, this does not necessarily mean that

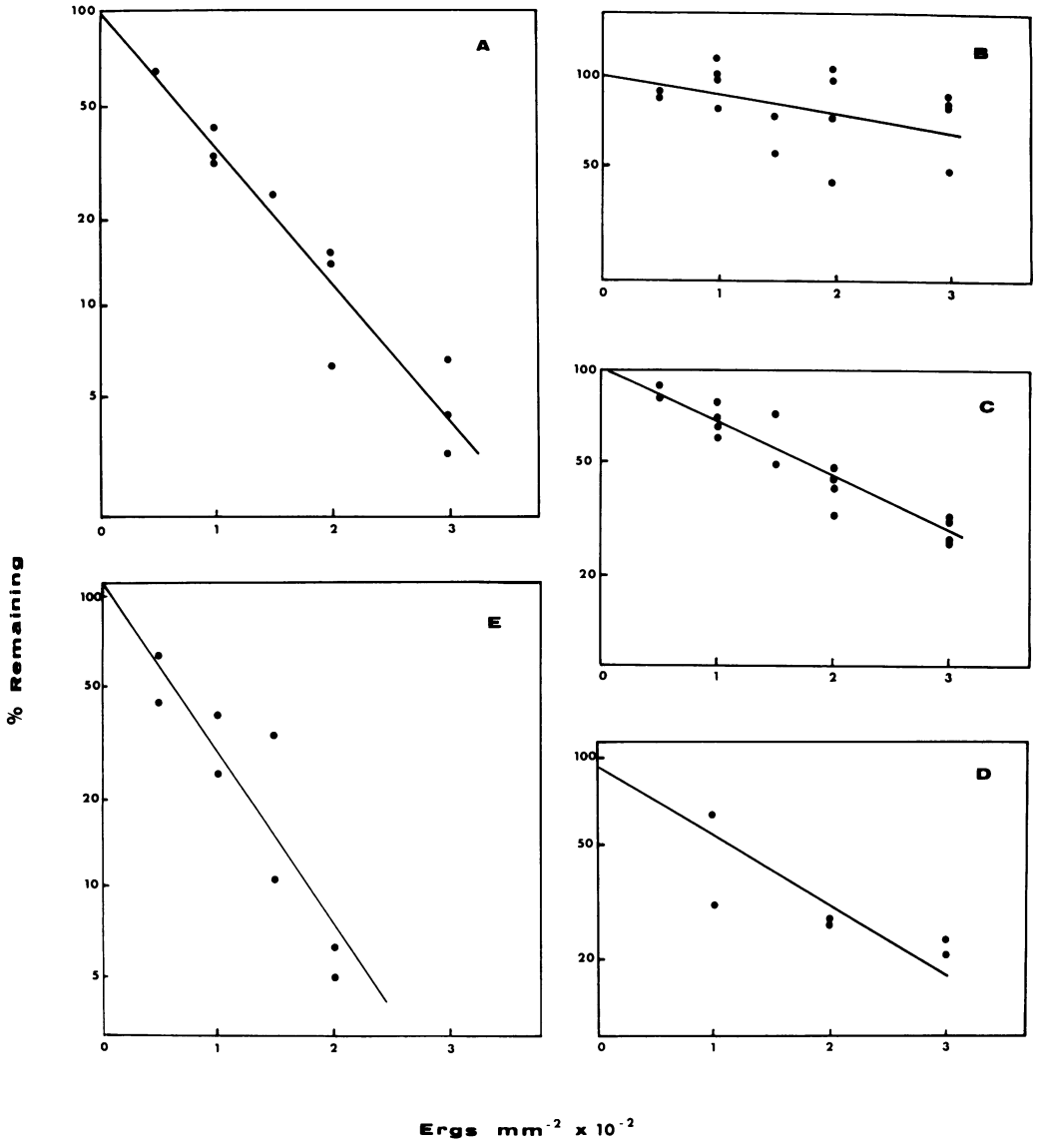

FIG. 4. *Dose response curves of infectivity and viral protein synthesis as a function of UV dose. See text for experimental details. (A) Infectivity. Points are the means of three experiments, except for doses of 50 and 150 ergs/mm², obtained from one experiment. (B) Protein N; (C) protein M_1; (D) protein M_2; (E) protein L. Data are from four experiments. In two, cellular protein synthesis was depressed by high-salt treatment (see Fig. 1). In the other two, synthesis was depressed by prior UV irradiation of cells (see Fig. 2). For proteins N and M_1 which are detectable with both methods, there are four points per dose (except for 50 and 150 ergs/mm², performed only with the second method). For L and M_2 which are detectable with only one method, there are two points per dose. Both methods yielded the same inactivation curves for proteins N and M_1. For doses higher than 300 ergs/mm², the intensity of viral bands was similar to background cell protein synthesis, and statistical variations became considerable. Experimental points were fitted by a least-squares analysis to obtain the regression curves and to calculate the correlation coefficients.*

this species is not synthesized during primary transcription. Indeed, it it known that high-salt treatments selectively depress the synthesis of this protein (12). Preirradiation of host cells could also depress G protein synthesis, because lesions in the cell membrane are induced. Even if protein G were synthesized, it would have been difficult to detect, because it migrates as a diffuse band in slab gel electophoresis.

Our results also suggest that the lethal effects

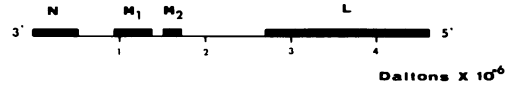

FIG. 5. *Hypothetical map of the rabies genome. The target sizes of proteins N, M_1, M_2, and L (calculated from Fig. 4) are shown, assuming a genome size of 4.6×10^6 daltons. The four cistrons are thus assumed to terminate at 5.1×10^5, 1.4×10^6, 1.7×10^6, and 4.6×10^6 daltons.*

of UV irradiation are due to a block of primary transcription, probably as a result of the enzyme being arrested on the template once it encounters a lesion.

Rabies proteins are not synthesized in equal quantities in infected cells. The extrapolation of the results of Madore and England (12) shows that protein N is synthesized in greater quantities than G, M_1, M_2, and L ($N > G > M_1 > M_2 > L$), which is consistent with the physical position of genes N, M_1, M_2, and L on the viral RNA molecule. It is possible that the regulation of protein synthesis could occur at the transcriptional level, e.g., by the unbinding of the enzyme before it reaches the 5′ end.

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