MOLECULAR AND CELLULAR BIOLOGY, Oct. 1984, p. 2231–2234
0270-7306/84/102231-04$02.00/0

Vol. 4, No. 10

Formation of Vesicular Stomatitis Virus Nucleocapsid from Cytoskeletal Framework-Bound N Protein: Possible Model for Structure Assembly

PRADEEP K. CHATTERJEE,[1]† MARGARITA M. CERVERA,[2] AND SHELDON PENMAN[1]*

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139,[1] and Instituto de Enzimologia, Arzobispo Morcillo, Madrid-32, Spain[2]

Received 21 June 1984/Accepted 17 July 1984

The pathway of vesicular stomatitis virus N protein from synthesis to assembly into capsids was studied by use of detergent extraction of infected HeLa cells together with protein cross-linking. One half of the newly synthesized N protein was extracted with the soluble cell proteins and, when cross-linked, never formed the N-N dimer characteristic of mature nucleocapsids. In contrast, the cytoskeleton-bound N protein first showed a diffuse spectrum of protein-protein cross-links but, after a lag of 40 min, assumed the cross-link pattern of N protein in nucleocapsids. The efficiency of forming N-N cross-linked dimers is the same for N protein on the skeleton as in nucleocapsids derived from mature virus, suggesting very similar configurations. However, the N protein bound on the skeletal framework formed several additional cross-links that were not found in mature virus and were apparently formed to cellular proteins estimated to be ca. approximately 46,000 and 60,000 in molecular weight.

The cytoskeletal framework of cultured mammalian cells is obtained by extraction with nonionic detergent under near-physiological conditions of pH and ionic strength. Soluble proteins and lipids are selectively removed, leaving the structural elements organized into a biochemically well-defined, intact entity (3, 4, 6, 11, 14, 15). The three-dimensional, anastomosing cytoplasmic filament networks composing the framework are best studied morphologically by embedment-free electron microscopy with either whole mounts or resin-free sections (5). The networks are regionally heterogeneous, with areas rich in microfilaments often found at the cell periphery and thicker fibers with attached polyribosomes localized nearer the nucleus. Numerous reports have shown that various aspects of virus metabolism are closely associated with these structural elements of the cell (2, 6, 10, 13, 16).

Little is known of the mechanisms by which these complex, heterogeneous networks are formed, but the pathways of structure formation may be more complicated than spontaneous self-assembly. One such pathway is suggested by the synthesis and assembly into a capsid of the N protein of vesicular stomatitis virus (VSV), a cytoplasmic virus composed of five proteins and a 42S negative-strand RNA (17). The formation of its capsid from the four internal proteins offers a very simple system for the study of structure assembly. Here we report the results of cross-linking analysis which suggest that only the skeleton-bound portion of the N protein enters into capsid formation and appears to do so without leaving cell structures. There was a significant lag between synthesis and final assembly of the skeleton-bound N protein, during which time its cross-linking neighbors changed markedly.

The partition of newly synthesized VSV proteins between the detergent-prepared cytoskeletal framework and the extracted soluble phase as a function of time was examined by briefly pulse-labeling (3 min) infected cells with [^{35}S]methionine and then chasing with cold methionine. Under the conditions of infection, only viral proteins are labeled. After various lengths of time, the cells were extracted with Triton X-100 in cytoskeleton buffer (100 mM NaCl, 3 mM $MgCl_2$, 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], pH 6.8, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100) (6) and the bound and soluble viral proteins were separately analyzed by gel electrophoresis (Fig. 1). The 3-min pulse labeled mostly complete polypeptides except for a ladder (Fig. 1, L) seen after 2 and 5 min of chase (see Fig. 2 and 3) and which appeared to be due to partially completed L chains which quickly entered into complete L protein at later chase times.

The L protein (polymerase) of VSV exhibited the simplest behavior. Essentially all of the L protein was bound to the skeleton immediately after its synthesis and remained so throughout the long chase. In contrast, only 50 to 60% of the other viral structural proteins, N (capsid), M (matrix), and NS (core), were bound, a proportion that remained constant throughout the chase. Finally, the transmembrane G (coat) protein was completely extracted by the detergent at all times.

The constancy of the bound fraction of viral proteins both before and during assembly into a capsid structure is most easily explained by the absence of significant exchange between soluble and structure-bound proteins, although presumably other explanations are possible. Earlier reports of a time-dependent partition between soluble and structure-bound capsid protein may reflect the very different extraction conditions used (8, 9).

The state of assembly of the viral nucleocapsid proteins bound to the cytoskeleton and of those in the soluble phase were investigated by using chemical cross-linking. L and NS proteins are relatively minor, and their cross-linking patterns were difficult to observe. M protein, which appears to be on the outside of the N-NS core in mature virus (7), seemed to associate with the nucleocapsid only just before budding. M protein did not form a significant number of

* Corresponding author.
† Present address: Molecular Biology Department, Princeton University, Princeton, NJ 08544.

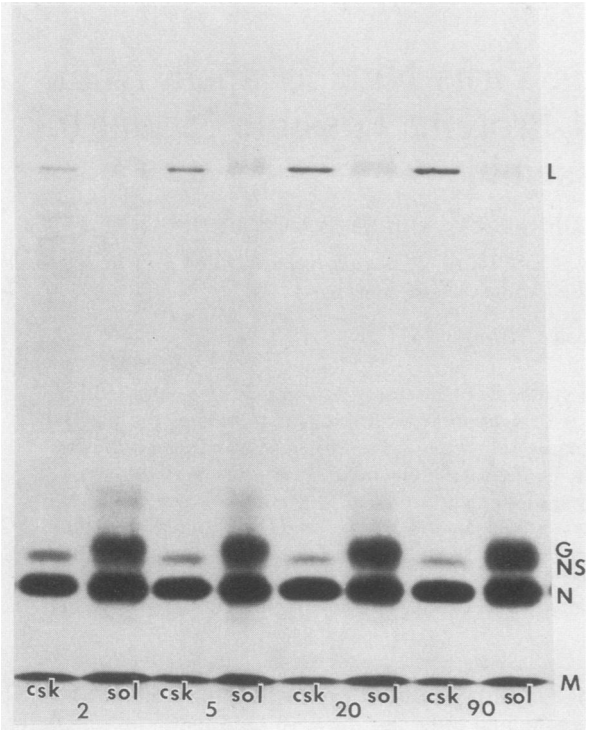

FIG. 1. Comparison of the cytoskeletal and soluble fractions at different times after pulse-labeling of VSV-infected cells. At 3 h after infection, cells were treated with actinomycin D for 0.5 h and pulse-labeled for 3 min. Equal numbers of cells were chilled at 2, 5, 20, and 90 min during the chase and fractionated into cytoskeleton and soluble phases as described in the text.

cross-links while in the cell, and there was no way to tell whether the capsid-associated M protein originated from the soluble or the bound state. However, N protein, the major constituent of the nucleocapsid, gave clear cross-linking patterns while bound to the cytoskeletal framework and was examined in detail.

The cross-linking data presented below follows the structure-bound N protein as it gradually took on nucleocapsid-like cross-links. At the same time, and in sharp contrast, the soluble proteins, including N protein, did not assemble into structures that were identifiable by cross-linking. The cross-linking agent used in these studies, dithiobis-(succinimidylpropionate), is extremely efficient, rapid, and reproducible in action (12).

The cross-linking results were obtained by using cytoskeletons and thus are free of adventitious binding of soluble proteins. The detergent-prepared cytoskeletons (6) were pelleted from the extraction buffer and gently suspended into cytoskeleton buffer containing 0.7 mM cross-linker at 0°C. The degree of cross-linking was carefully controlled and kept low in the following experiments so that two-protein cross-links would predominate. After cross-linking took place for 2 min at 0°C, excess cross-linker was quenched with ammonium acetate. Free sulphydryl groups in proteins were blocked with *N*-ethylmaleimide, and the cytoskeletons were then selectively solubilized away from the nuclei in RSB buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-Cl, pH 7.4) containing 0.5% deoxycholate and 1% Tween 40 (6). Higher levels of cross-linking drastically reduced the recovery of viral and cellular proteins at this stage of selective solubilization. Disuccinimidyl-suberate, a cross-linker not containing a disulfide bond, gave similar results in one-dimensional polyacrylamide gel electrophoresis. The pattern of cross-linked proteins obtained from purified complete virions (data not shown) agreed well with previously published data (7) in which three different cross-linking agents gave similar results.

Figure 2 shows the results of cross-linking the cytoskeleton from pulse-labeled, VSV-infected cells after different chase intervals. Although much of the newly synthesized N protein was bound immediately and remained associated with the cytostructure, its cross-linking pattern indicates that it assembled into a capsid-like particle only after about 40 min. Initially, N protein had a very different set of cross-linking neighbors, which changed to a capsid-like form during the chase. The cluster of faint bands (Fig. 2, 1) in the 2-min chase cross-linked (lane 2x) were absent from the non-cross-linked lane (2u) and, unlike the ladder of bands below L in lane 2u, formed off-diagonal spots in two-dimensional analysis (Fig. 2B).

The identity of the components of the cross-link bands was confirmed by polyacrylamide gel electrophoretic analysis in a second dimension after cleavage of the cross-linker with β-mercaptoethanol (7, 12). The cross-linked bands in one dimension (Fig. 2A) at late chase times or the off-diagonal spots in a two-dimensional gel (Fig. 2B) were compared to the pattern obtained from cross-linking isolated complete virions or detergent-stripped nucleocapsids (Fig. 3; two-dimensional gel not shown). It is clear that some spots (in two dimensions) and bands (in one dimension), such as 8 and probably 7 (see Figure 2), in these electropherograms arose from intracapsid cross-links, but several, such as 9, 6, and 4, were clearly absent from the nucleocapsid pattern and were probably formed by viral capsid proteins also cross-linking to cytoskeletal elements. The major cross-linked band (Fig. 2A, band 8) which appears in the cytoskeleton proteins after long chase times was the N-N dimer and was superimposable with the major off-diagonal spot from cross-linked isolated nucleocapsids on a two-dimensional gel (data not shown). Since the efficiency of the dimer cross-link was extremely sensitive to inter-protein distance, it is likely that the N-N cross-link is very characteristic of nucleocapsids or of some intermediate structures having a close resemblance to a nucleocapsid. Nearly the same amount of N-N dimer was formed, for a given degree of cross-linking, from virions, isolated nucleocapsids, and most importantly, from skeleton-bound N protein when the latter had had sufficient time to assume its final state (i.e., at least 40 min after pulse-labeling). We estimated crudely the molecular weight of some of the putative cytoskeletal proteins cross-linked to the viral ones as being 46,000 and 60,000 (Fig. 3, arrows).

Only the cytoskeleton-bound proteins appeared to enter into nucleocapsids, and not even all of these did so. The core protein NS was overproduced, and the amount of this protein bound to the cytoskeleton relative to N and M was markedly greater than that found in isolated virions or nucleocapsids. Thus, much of even the skeleton-bound NS did not enter a nucleocapsid. L protein appeared to be present on the cytoskeleton in amounts relatively greater than in the final virion, much like NS but not nearly to the same degree. Comparison of lanes csk x with lanes NcX (nucleocapsid) and Vx (whole virus) in Fig. 3 shows that about one-half of L protein on the skeleton did not form detectable cross-links, whereas all of L protein in the isolated virion or nucleocapsid did. This is consistent with the rough estimate that there is about twice as much L

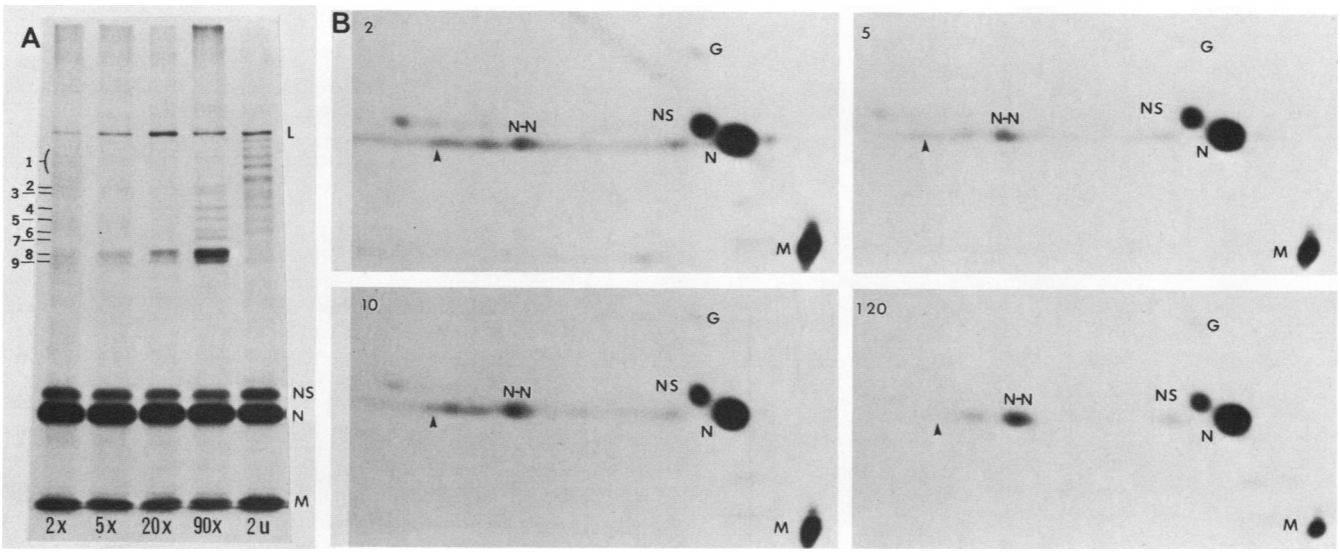

FIG. 2. Polyacrylamide gel electrophoretic analysis of the cytoskeleton cross-linking patterns at different chase intervals. Equal numbers of pulse-labeled, VSV-infected cells were chilled in cold phosphate-buffered saline at 2, 5, 20, and 90 min of chase and detergent extracted, and the cytoskeletons were cross-linked after removal of the soluble phase. 2u, Non-cross-linked cytoskeleton after 2 min of chase. (B) Two-dimensional gel analysis of the cytoskeleton cross-linking patterns at 2, 5, 10, and 120 min after pulse-labeling VSV-infected cells. The 2-min gel was exposed longer to emphasize the diffuse but distinct cross-linked spots indicated by the arrowhead. These disappeared at 120 min, suggesting that the specific association of N at the earliest times is different from those at later times.

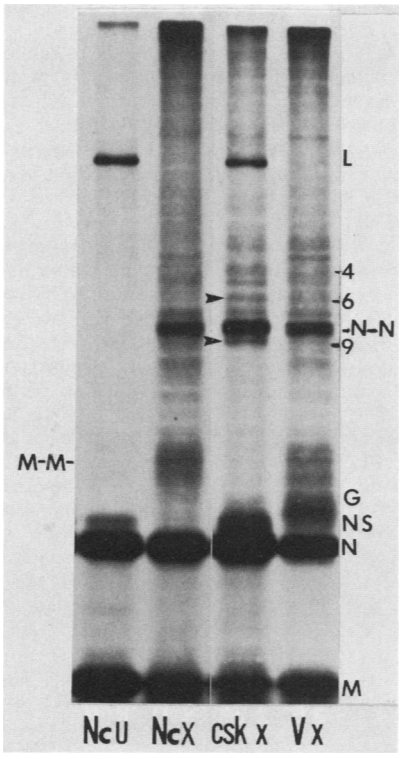

FIG. 3. Cross-linking patterns of purified VSV, isolated nucleocapsids, and cytoskeleton-bound VSV proteins. NcU, Non-cross-linked nucleocapsid; NcX, cross-linked nucleocapsid; Vx, cross-linked virus; Csk x, cross-linked cytoskeleton at 60 min of chase; N-N, cross-linked dimer of N protein; M-M, cross-linked dimer of M protein. The arrowheads indicate the position of some cross-linked bands present in the cross-linked cytoskeleton but not in the cross-linked nucleocapsid. (The four lanes are from the same gel.)

protein on the skeleton than in the virion relative to N protein. It should be noted also that the matrix protein M bound to the skeleton did not form the M-M dimer cross-link (cf. lane csk x with lanes NcX and Vx in Fig. 3), nor did it give any other cross-links by the absence of off-diagonal spots, as seen in the two-dimensional electropherogram (see Fig. 2B). M did give strong cross-linked spots in the mature virus or in the nucleocapsids prepared by detergent extraction of virions when analyzed in two-dimensional gels (data not shown). Results from other laboratories have indicated the association of M with the maturing capsid just before budding (1). Thus, the absence of M cross-links is not unexpected.

Although the soluble phase contained substantial quantities of the viral proteins, these, in sharp contrast to the skeleton-bound proteins, did not form any specific cross-links. The pattern obtained by cross-linking proteins in the soluble phase is shown in Fig. 4 and compared with the very different result for proteins on the cytoskeleton. No identifiable cross-linked bands were seen. Exactly the same absence of pattern was seen at 2, 5, 10, and 60 min of chase. As a control to show that virion-specific cross-links would be formed if nucleocapsids were present, a small amount of VSV was added to the soluble proteins; this gave a cross-linked pattern much the same as that of virions suspended in buffer.

We could not definitively prove the absence of exchange between the soluble and cytoskeleton-bound proteins. However, the assembly of N into a cross-linkable structure did not appear to be a stochastic process, but proceeded slowly at first and then rapidly, reaching a maximum after about 40 min of pulse-label. This is certainly not the behavior one would expect if the cross-linkable N protein were being withdrawn from a random, soluble pool. Also, the soluble viral proteins appeared never to assemble into cross-linkable structures.

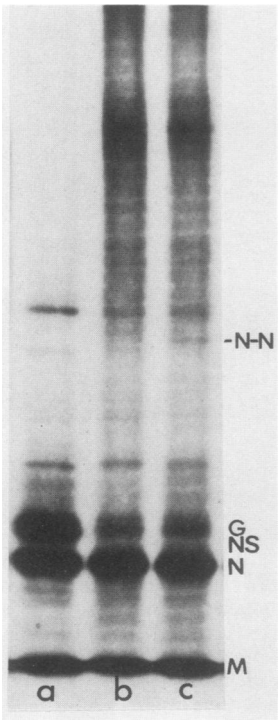

FIG. 4. Cross-linking of soluble-phase VSV proteins. Lanes a and b, Non-cross-linked and cross-linked soluble phases, respectively, from the 60-min chase sample of VSV-infected, pulse-labeled cells. The identical pattern was obtained at 2, 5, and 10 of chase. Lane c, Cross-linking of 60-min chase soluble phase containing exogenously added VSV to one-sixth the total counts, with equal amount of cross-linker as in lane b.

There was a slight increase in the amount of N protein associated with the skeleton after long chase times. Though we cannot rule out directly the possibility of a tiny fraction of N from the soluble phase associating with the skeleton with time, this very small increase may reflect our inability to completely stabilize against extraction the structures initially binding N protein. It may be noted that, in other studies (8, 9) in which harsher extraction conditions were used, significantly more N protein was solubilized at early times after labeling as compared with later times.

LITERATURE CITED

1. **Atkinson, P. H., S. A. Moyer, and D. F. Summers.** 1976. Assembly of vesicular stomatitis virus glycoprotein and matrix protein into HeLa cell plasma membranes. J. Mol. Biol. **102**:613–631.
2. **Ben-Ze'ev, A., M. Horowitz, H. Skolnick, R. Abulafia, O. Laub, and Y. Aloni.** 1981. The metabolism of SV40 RNA is associated with the cytoskeletal framework. Virology **111**:475–487.
3. **Blikstad, I., and E. Lazarides.** 1983. Synthesis of Spectrin in avian erythroid cells: associated of nascent polypeptide chains with the cytoskeleton. Proc. Natl. Acad. Sci. U.S.A. **80**:2637–2641.
4. **Brown, S., W. Levinson, and J. A. Spudich.** 1976. Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. J. Supramol. Struct. **5**:119–130.
5. **Capco, D. G., G. Krochmalnic, and S. Penman.** 1984. A new method of preparing embedment-free sections for TEM: application to the cytoskeletal framework and other three-dimensional networks. J. Cell Biol. **98**:1878–1885.
6. **Cervera, M., G. Dreyfuss, and S. Penman.** 1981. Messenger RNA is translated when associated with the cytoskeletal framework in normal and VSV infected HeLa Cells. Cell **23**:113–120.
7. **Dubovi, E. J., and R. R. Wagner.** 1977. Spatial relationships of the proteins of vesicular stomatitis virus: induction of reversible oligomers by cleavable protein cross-linkers and oxidation. J. Virol. **22**:500–509.
8. **Hsu, C-H., D. W. Kingsbury, and K. G. Murti.** 1979. Assembly of vesicular stomatitis virus nucleocapsids in vivo: a kinetic analysis. J. Virol. **32**:304–313.
9. **Knipe, D. M., D. Baltimore, and H. F. Lodish.** 1977. Separate pathways of maturation of the major structural proteins of vesicular stomatitis virus. J. Virol. **21**:1128–1139.
10. **Lenk, R., and S. Penman.** 1979. The cytoskeletal framework and poliovirus metabolism. Cell **16**:289–301.
11. **Lenk, R., L. Ransom, Y. Kaufman, and S. Penman.** 1977. A cytoskeletal structure with associated polyribosomes obtained from HeLa cells. Cell **10**:67–78.
12. **Lomant, A. J., and G. Fairbanks.** 1976. Chemical probes of extended biological structures: synthesis of properties of the cleavable protein crosslinking reagent ^{35}S dithiobis-(succinimidyl propionate). J. Mol. Biol. **104**:243–261.
13. **Murti, K. G., and R. Goorha.** 1983. Interaction of frog virus-3 with the cytoskeleton. 1. Altered organization of microtubules, intermediate filaments, and microfilaments. J. Cell Biol. **96**:1248–1250.
14. **Osborn, M., and K. Weber.** 1977. The detergent-resistent cytoskeleton of tissue culture cells includes the nucleus and the microfilament bundles. Exp. Cell Res. **106**:339–349.
15. **Penman, S., A. Fulton, D. Capco, A. Ben-Ze'ev, S. Wittelsberger, and C. F. Tse.** 1982. Cytoplasmic and nuclear architecture in cells and tissue: form, functions and mode of assembly. Cold Spring Harbor Symp. Quant. Biol. **46**:1013–1027.
16. **Quinlan, M. P., and D. M. Knipe.** 1983. Nuclear localization of herpesvirus proteins: potential role for the cellular framework. Mol. Cell. Biol. **3**:315–324.
17. **Wagner, R. R.** 1975. Reproduction of rhabdovirus, p. 1–93. *In* H. Frankel Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 4. Plenum Press, New York.