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Intranuclear topological distribution of HIV-1 trans-activators

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Subcellular localization of human immunodeficiency virus type I (HIV-1) Tat and Rev was examined using a confocal laser scanning microscope (CLSM). In transfected COS-7 cells, Tat resided exclusively in the perinucleolar region, while Rev infiltrated fully into the nucleoli. The chimeric Tat in which the nucleolar targeting signal was replaced by that of Rev, which retains *trans*-acting activity of Tat, remained still in the perinucleolar region as wild-type Tat. Perinucleolar distribution of Tat protein suggests the existence of a novel nucleolar architecture that affects transcription.

Nucleolus; Nucleolar targeting signal; NOS; Tat; Rev

1. INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), encodes several regulatory proteins which are of importance for regulation of viral replication, in addition to the characteristic retroviral *gag*, *pol* and *env* gene products. Two of these, termed Tat and Rev, are essential trans-activators of viral gene expression [1,2] and thus have been considered to be attractive targets for prophylactic and therapeutic intervention in AIDS. Rev is a small protein of 116 amino acids with an electrophoretic mobility of approximately 19 kDa on sodium dodecyl sulfate (SDS) gels. Rev is thought to function at a posttranscriptional level to permit synthesis of the viral structural proteins by increasing the stability of the unspliced viral mRNAs and/or promoting the transport of the viral mRNAs [3,4]. Tat is also a small protein of 86 amino acids which has a molecular weight of about 16 kDa. Tat is an RNA sequence-specific trans-activator that has been shown to interact directly with its target sequence, an RNA stem-loop structure located at the 5′ end of all HIV-1 transcripts, referred to as TAR (the Tat-responsive element) [5]. Although much is known about Tat and its recognition sequence TAR, the mechanism of action of Tat remains controversial, with both transcriptional and posttranscriptional mechanisms being considered [6,7]. Both Tat and Rev are known to be located predominantly in cell nucleoli by the transfection experiments [6,8,9], and both have a nucleolar targeting signal (NOS) [10,11].

In general, a correlation exists between nucleolar accumulation of Rev and its function [8]. On the other hand, the physiological relevance of nucleolar accumulation to Tat function remains to be determined. We examined the subcellular localization of these two trans-activators within transfected COS-7 cells. Confocal laser scanning microscope (CLSM) was employed to obtain serial images of tomographic optical slices (microtomographic images) with high resolution of the inner structures of the cells [12,13].

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2. MATERIALS AND METHODS

2.1. *Cells*

COS-7 (a monkey kidney cell line) cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and served for DNA transfection experiments.

2.2. *Plasmids*

A eukaryotic *rev* expression plasmid, termed pH2rev, was constructed by inserting a DNA fragment containing *rev* cDNA into pKCRH2 which has the SV40 early promoter and poly(A) sequence [10]. A eukaryotic *tat* expression plasmid, termed pH2Ftat, contains a DNA fragment coding for 86 intact amino acids of Tat [14]. A mutant *tat* expression plasmid, pH2tat-AR, was constructed by replacing the sequence coding for the highly basic region of Tat with the highly basic-region DNA sequence of the *rev* gene [14]. The mutant protein, H2tat-AR, stimulated CAT activity to 30–40% of the level directed by the wild-type Tat protein [14].

2.3. *DNA transfection*

COS-7 cells were seeded on glass coverslips in flat-bottom cell wells (1×10^5/well, well diamter: 35 mm) 1 day before transfection. Two micrograms of plasmid DNA was transfected into the cells by the calcium phosphate method [9] with a glycerol shock 4 h after transfection.

2.4. *Indirect immunofluorescence*

Sixty-six hours after transfection, COS-7 cells were fixed with 3.5% formaldehyde/PBS, permeabilized with 0.1% Nonidet P-40/PBS, and incubated with an affinity-purified rabbit anti-Rev C-terminal polyclonal antibody or polyclonal rabbit antiserum against N-terminal

synthetic polypeptides of Tat (1–63 amino-acid residues), then stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody. Cells were simultaneously stained for the nucleoli with serum from a patient with progressive systemic sclerosis as anti-nucleolus antibody [16], followed by visualization with tetramethylrhodamine-conjugated anti-human IgG antibody. All specimens were finally mounted with 90% glycerin-PBS containing 0.1%*p*-phenylenediamine.

2.5. *Confocal laser scanning microscope (CLSM)*

The confocal images (512 × 512 × 8-bit) were collected with a laser scanning microscope system (Olympus LSM-GB) under the following conditions: object lens, Olympus SPlan-Apo × 100 (NA=1.4); zoom ratio, × 4.0; scan speed, 2 s (Fig. 1d) or 1 s (Fig. 1b) per frame, and were transferred to an image processor (Olympus CIA) for averaging 8 times in Fig. 1d, or 16 times in Fig. 1b. The step distance between

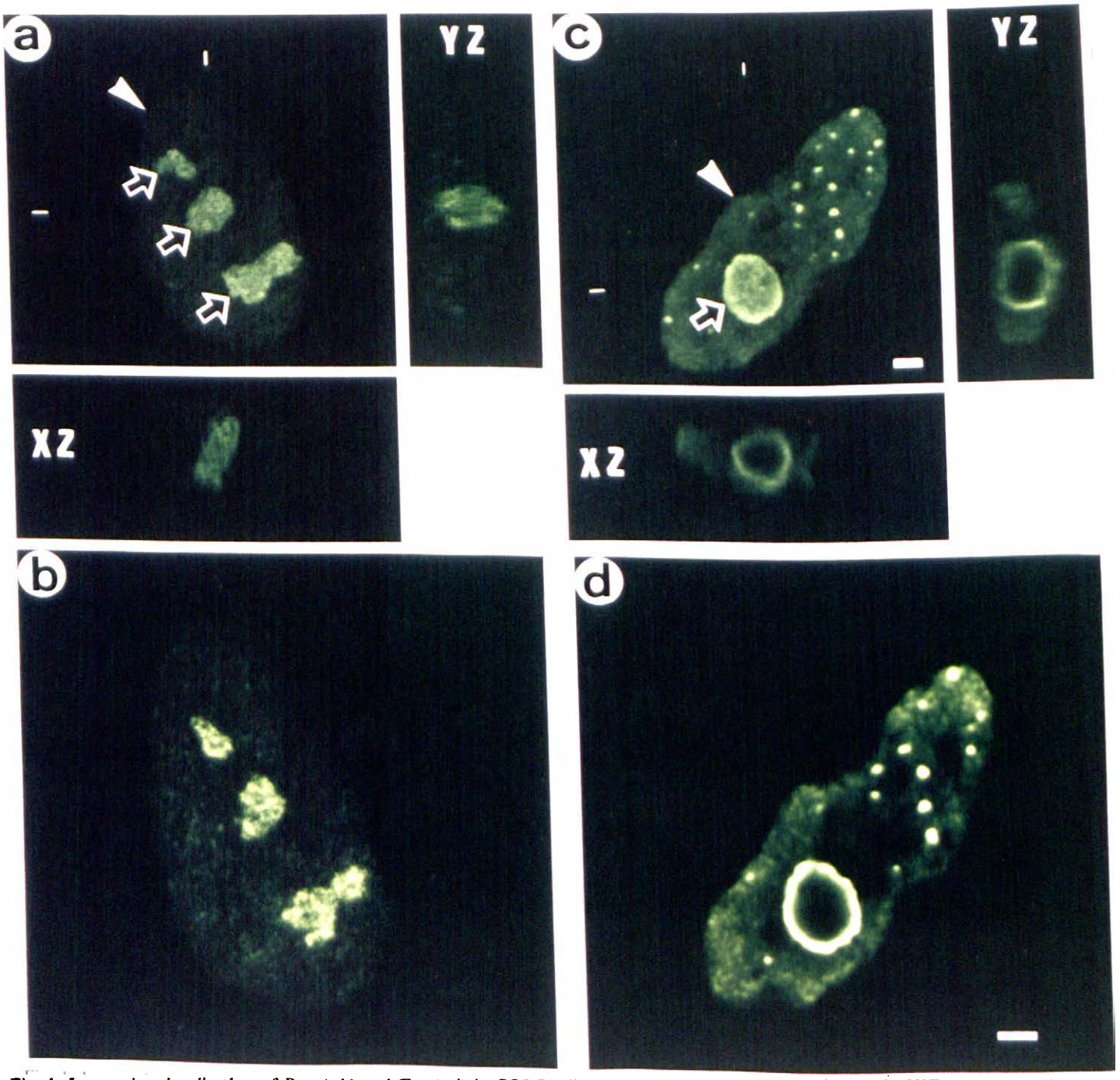

Fig. 1. Intranuclear localization of Rev (a,b) and Tat (c,d) in COS-7 cells 66 h after transfection of pH2rev and pH2Ftat. In (a) and (c), extended-focus images and cross-sectional images along the horizontal (X) axis and vertical (Y) axis obtained from the serial confocal images (18 images in (a), 20 images in (c)) are shown. The short lines at the top and the left side of (a) and (c) represent X and Y coordinates of the cross-sectional images, respectively. The open arrow indicates the nucleolus and the arrowhead indicates the nucleus. Microtomographic images of the cells corresponding to those in (a) and (c) are shown in (b) and (d), respectively. The white bars represent 2 μm.

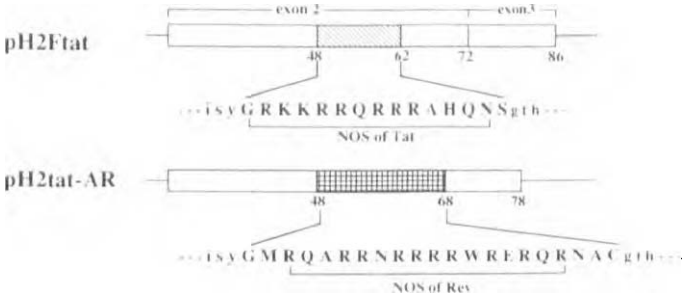

Fig. 2. Schematic representation of a *tat* expression plasmid, pH2Ftat, and its mutant, pH2tat-AR. The sequence ^{48}GRKKRRQRRRA-HQN^{61}, present in H2Ftat, is the highly basic region of Tat. The sequence ^{50}RQARRNRRRRWRERQR^{65}, present in H2tat-AR, is derived from the HIV-1 *rev* gene.

each image was 0.4 μm. During serial optical sectioning, fine control of sample movement was accomplished by a piezoelectric translator and its controller which could achieve reproducible movement of the sample stage at an accuracy of within 40 nm.

3. RESULTS AND DISCUSSION

In *rev*-transfected cells, fluorescence was observed in the whole part of the nucleoli 66 h after transfection (Fig. 1a,b). By contrast, Tat accumulated solely in the perinucleolar region (Fig. 1c,d), albeit with an arginine-rich motif similar to that of Rev [17]. Nucleoplasmic staining with speckled pattern was also observed in some *tat*-transfected cells (Fig. 1c,d). Both Rev- and Tat-expressing cells were detected in 10–15% of the transfected cells by indirect immunofluorescence, and there was no difference in fluorescence intensity between them. It is, therefore, unlikely that the topological difference of the virus trans-activators in the nuclei results simply from indiscriminate binding of their basic amino acid residues with rRNAs.

The nucleolus is not an isolated floating organelle in the nucleoplasm but a structure linked to both the chromosomes and the nuclear envelope [18], and a relationship between the position of the nucleolus near the nuclear envelope and the export of the nucleolar RNA towards the cytoplasm has been suggested [18,19]. Furthermore, major nucleolar proteins, nucleolin and No38, have been shown to shuttle between nucleus and cytoplasm, suggesting a role for these nucleolar proteins in the nucleocytoplasmic transport of ribosomal components [20]. Rev is thought to operate at posttranscrip-

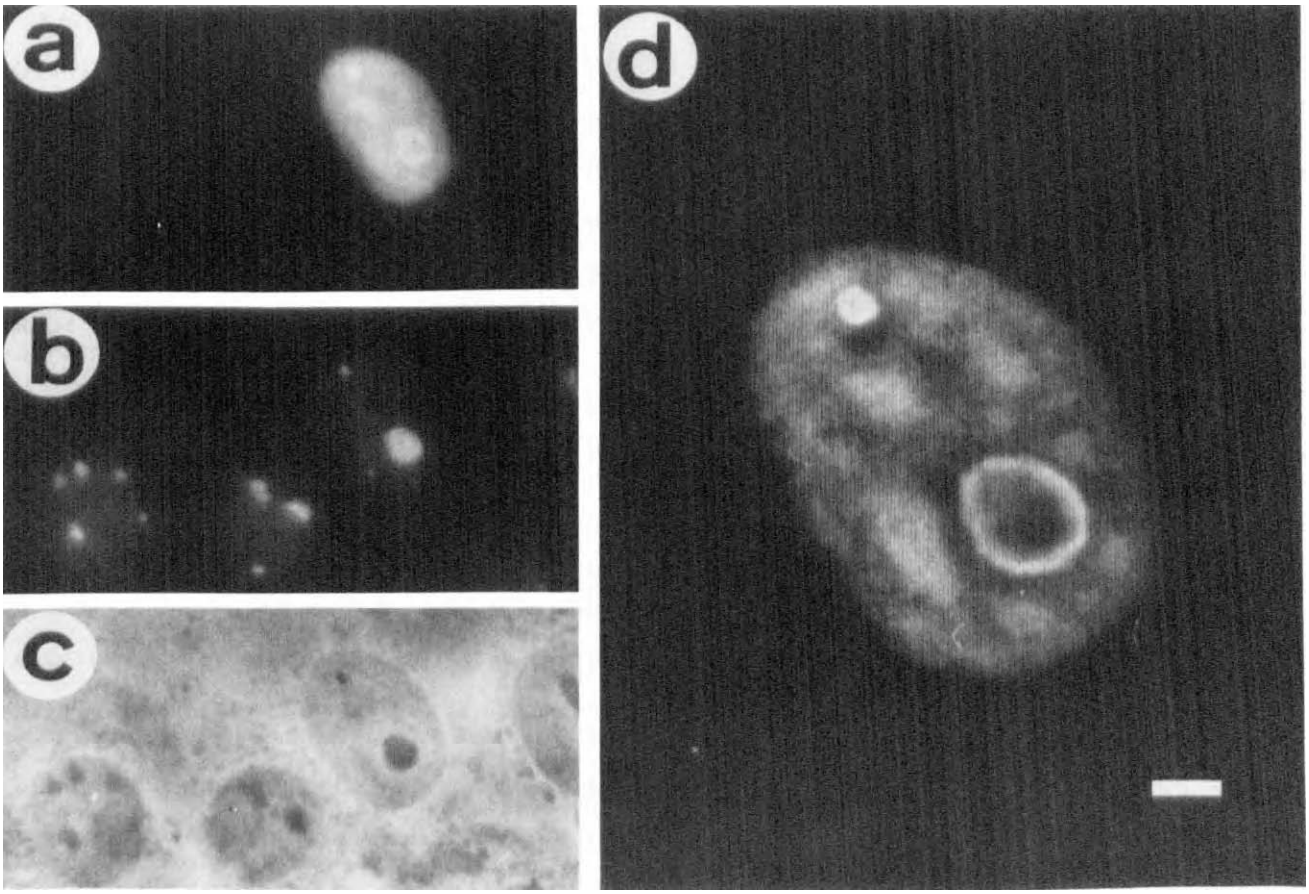

Fig. 3. Subcellular localization of Tat mutant protein, H2tat-AR. Transfected COS-7 cells were doubly stained with anti-Tat antibody (a,d) and serum from a patient with progressive systemic sclerosis as anti-nucleolus antibody (b) [26]. Immunocomplexes were visualized by FITC-conjugated anti-rabbit IgG antibody (a,d) and tetramethylrhodamine-conjugated anti-human IgG antibody (b). Microtomographic image by CLSM is shown in (d). Phase-contrast micrograph of the same field as (a) and (b) is shown in (c). The bar in (d) represents 2 μm.

tional stages. Nucleolar function(s) in posttranscriptional regulation should further be investigated.

Previous studies using transfected cells have demonstrated the nuclear/nucleolar localization of Tat by using conventional microscopes. However, the significance of nucleolar localization of Tat has not been verified yet. In the study using transfected cells, a *trans*-dominant mutant of Tat inhibited Tat function by preventing nucleolar migration [21]. In the other study, however, endocytosed rhodamine-labeled Tat was not located in the nucleoli, albeit functional in an assay system [22], suggesting that nucleolar localization per se is not essential for transactivation by Tat. By using CLSM, we demonstrated the specific perinucleolar accumulation of Tat in the transfected COS-7 cells, indicating that Tat need not be destined for inside the nucleoli. However, its specific accumulation in the perinucleolar region suggests that Tat is assigned with some relation to the nucleoli. To elucidate the significance of perinucleolar localization of Tat for its function, we examined subcellular localization of a mutant Tat protein, H2tat-AR [14], which was made by replacement of the highly basic region of Tat with that of Rev (Fig. 2). This mutant protein was previously shown to retain 30–40% *trans*-acting activity as compared with wild-type Tat protein [14]. As shown in Fig. 3, the Tat–Rev fusion protein was less predominantly located in the perinucleolar region than wild-type Tat protein. This finding suggests the necessity of Tat structure with NOS for perinucleolar localization and function.

Tat transactivates viral gene expression at the level of both transcriptional initiation and elongation from the HIV-1 long terminal repeat [7,23,24]. It is, however, still the subject under discussion whether Tat increases the expression of viral genes at a posttranscriptional stage. Direct evidence for a posttranscriptional role of Tat was obtained from the experiments of microinjection into Xenopus oocytes [25]. In primate cells permissive for viral replication, however, Tat exerts its effect primarily at the level of transcription [26]. Recently, Carter et al. [27] reported that nuclear poly(A) RNA was found concentrated primarily within several discrete domains which often surrounded the nucleoli. Their data also suggest that these poly(A) RNA regions are transcription sites. Considering in connection with our results that Tat is predominantly located in the perinucleolar region, there is a possibility that Tat may transactivate viral gene expression in this region. The nucleolus is not separated by any membranous structure from the surrounding nucleoplasm, and no unique structure has been identified in the boundary between the nucleolus and the nucleoplasm. The perinucleolar accumulation of Tat could reflect the novel nucleolar architecture where Tat exerts its *trans*-acting effect. To investigate the relevance of perinucleolar localization of Tat to its function may help to clarify the mechanism by which Tat transactivates gene expression, and should provide a clue for understanding hitherto unknown organization of the nucleolus.

The nucleolus is well known to be an organelle of ribosome biogenesis. However, other functions are not fully understood as yet, and we know very little about how certain proteins are imported into the nucleolus. Human T-cell leukemia virus type I (HTLV-I) Rex protein, which may function in a manner similar to Rev, has been shown to be located in the nucleolus [16,28,29]. The study of HIV-1 Tat, Rev, and HTLV-I Rex will shed light on an intriguing function(s) of the nucleolus.

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REFERENCES

[1] Sodroski, J., Goh, W.C., Rosen, C.A., Dayton, A.I., Terwillinger, E. and Haseltine, W. (1986) Nature 321, 412–417.
[2] Dayton, A.I., Sodroski, J.G., Rosen, C.A., Goh, W.C. and Haseltine, W.A. (1986) Cell 44, 941–947.
[3] Omitted.
[4] Felber, B., Hadzopoulou-Cladaras, M., Cladaras, C., Copeland, T. and Pavlakis, G. (1989) Proc. Natl. Acad. Sci. USA 86, 1495–1499.
[5] Rosen, C.A., Sodroski, J.G. and Haseltine, W.A. (1985) Cell 41, 813–823.
[6] Hauber, J., Perkins, A., Heimer, E.P. and Cullen, B.R. (1987) Proc. Natl. Acad. Sci. USA 84, 6364–6368.
[7] Muesing, M.A., Smith, D.H. and Capon, D. (1987) Cell 48, 691–701.
[8] Cochrane, A.W., Perkins, A. and Rosen, C.A. (1990) J. Virol. 64, 881–885.
[9] Malim, M.H., Böhnlein, S., Hauber, J. and Cullen, B.R. (1989) Cell 58, 205–214.
[10] Kubota, S., Siomi, H., Satoh, T., Endo, S., Maki, M. and Hatanaka, M. (1989) Biochem. Biophys. Res. Commun. 162, 963–970.
[11] Siomi, H., Shida, H., Maki, M. and Hatanaka, M. (1990) J. Virol. 64, 1803–1807.
[12] Takamatsu, T. and Fujita, S. (1988) J. Microsc. 149, 167–174.
[13] Minamikawa, T., Takamatsu, T. and Fujita, S. (1991) Acta Histochem. Cytochem. 24, 55–60.
[14] Endo, S., Kubota, S., Siomi, H., Adachi, A., Oroszlan, S., Maki, M. and Hatanaka, M. (1989) Virus Genes 3, 99–110.
[15] Graham, F. and Van der Eb, A. (1973) Virology 52, 456–467.
[16] Siomi, H., Shida, H., Nam, S.H., Nosaka, T., Maki, M. and Hatanaka, M. (1988) Cell 55, 197–209.
[17] Lazinski, D., Grzadzielska, E. and Das, A. (1989) Cell 59, 207–218.
[18] Bourgeois, C.A. and Hubert, J. (1988) Int. Rev. Cytol. 111, 1–52.
[19] Hadjiolov, A.A. (1985) The Nucleolus and Ribosome Biogenesis, Springer, New York.
[20] Borer, R.A., Lehner, C.F., Eppenberger, H.M. and Nigg, E.A. (1989) Cell 56, 379–390.
[21] Pearson, L., Garcia, J., Wu, F., Modesti, N., Nelson, J. and Gaynor, R. (1990) Proc. Natl. Acad. Sci. USA 87, 5079–5083.
[22] Mann, D.A. and Frankel, A.D. (1991) EMBO J. 10, 1733–1739.
[23] Peterlin, B.M., Luciw, P.A., Barr, P.J. and Walker, M.D. (1986) Proc. Natl. Acad. Sci. USA 183, 9734–9738.
[24] Kao, S.Y., Calman, A.F., Luciw, P.A. and Peterlin, B.M. (1987) Nature 330, 489–493.

[25] Braddock, M., Chambers, A., Wilson, W., Esnouf, M.P., Adams, S.E., Kingsman, A.J. and Kingsman, S.M. (1989) Cell 58, 269–279.
[26] Chin, D.J., Selby, M.J. and Peterlin, B.M. (1991) J. Virol. 65, 1758–1764.
[27] Carter, K.C., Taneja, K.L. and Lawrence, J.B. (1991) J. Cell Biol. 115, 1191–1202.
[28] Nosaka, T., Siomi, H., Adachi, Y., Ishibashi, M., Kubota, S., Maki, M. and Hatanaka, M. (1989) Proc. Natl. Acad. Sci. USA 86, 9798–9802.
[29] Kalland, K.H., Langhoff, E., Bos, H.J., Göttlinger, H. and Haseltine, N.A. (1991) New Biol. 3, 389–397.