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Expression kinetics and subcellular localization of HIV-1 regulatory proteins Nef, Tat and Rev in acutely and chronically infected lymphoid cell lines

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Summary. Information concerning the expression kinetics and subcellular localization of HIV regulatory proteins is of importance in understanding the viral pathogenesis and may be relevant for drug and vaccine development, as well. We have used combined immunocytochemistry and in situ hybridization to study firstly, the order of expression of regulatory HIV-1 proteins Nef, Rev and Tat in relation to non-spliced and spliced mRNA expression and secondly, the subcellular localization of these proteins in acutely and chronically infected human T-cell lines. We used monoclonal antibodies against HIV-1 Nef, Tat, Rev and gp160, and RNA probes reacting either with all mRNAs (nef) or only with the full-length mRNA (gag-pol). In acutely infected MT-4 and H9 cells, four distinct phases of infection could be defined. In the first phase lasting from 0 to 6 h post-infection, only incoming virus could be demonstrated by gp160 immunocytochemistry. During the second, regulatory phase (6–9 h), abundant cytoplasmic expression of Nef, Rev and Tat proteins and a positive in situ RNA hybridization with the nef probe was seen, while the in situ hybridization with full-length mRNA probe and immunohistochemistry for gp160 were still negative. The productive phase (12–48 h) was characterized by abundant expression of full-length mRNA and gp160, and by the nuclear localization of Nef and Tat proteins. In contrast, an antibody that recognized the RRE binding region of the Rev protein localized Rev in the cytoplasm both during the regulatory and productive phase. During the fourth, cytopathic phase, the expression of mRNA or viral proteins decreased and the regulatory proteins studied were again mainly localized in the cytoplasm. Based on the results, we speculate that HIV Nef may function as a nuclear factor, and that Tat is possibly bound by cellular proteins before its transport to the nucleus.

Introduction

HIV-1 encodes at least seven regulatory and auxillary gene products in addition to viral structural proteins (reviewed in [51]. The molecular biology of some of these regulatory genes, like tat and rev, and the function of the corresponding proteins has been characterized to great extent (reviewed in [8]). Tat and Rev proteins are essential for viral replication both in vitro and in vivo. Tat mainly acts through increasing the rate of transcription from the HIV LTR 3 [22]. Recently, it has been found that cellular proteins (transcription factors, protein kinase) may bind to Tat [23, 40, 50], to its target structure TAR [15, 49] or to sites upstream of TAR [25], and thus cooperate in the overall transactivation process. Rev protein functions as a post-transcriptional regulator by inducing a shift from the expression of early, multiply spliced mRNA to mRNA for viral structural proteins [8, 9, 11, 34].

The role of Nef protein has remained controversial. Nef protein is not essential for viral growth [2, 12, 28, 33, 41, 52], but the fact that the nef reading frame is open and highly conserved among primate lentiviruses [39] suggests a pivotal role for nef in the viral life cycle. When first identified, Nef protein was thought to act as a negative regulator of HIV replication by inhibiting LTR-regulated gene expression [2, 36, 41]. It was shown that mutations in nef lead to more efficient viral replication [2, 19, 33, 41, 52], and, more recently, it has been proposed that Nef aids in the maintenance of HIV latency by downregulating the binding of proliferation-associated DNA-binding factors [18] and by inhibiting the induction of NF-kB DNA-binding activity [42]. Despite earlier controversial reports [7, 14, 19] it has now become evident that Nef also downregulates the expression of CD4 molecule on cell surface [4, 16, 24], possibly through intracellular sequestering. However, in vivo, Nef seems to be required for full pathogenic potential and maintenance of high virus load in the SIV model [29], and at least some natural Nef proteins accelerate virus production in primary human lymphocytes [10, 55] and in T-cell lines [55].

We previously found that Nef proteins from several HIV isolates are expressed in two isomorphic forms and that diverse HIV isolates show phenotypic variation in Nef expression as defined with a panel of monoclonal antibodies [43]. We also showed that the Nef protein is mainly localized in the cytoplasm within the Golgi complex, but that Nef may temporarily be expressed also in the nucleus [32, 43].

In the present work we studied the early expression kinetics and subcellular localization of HIV-1 structural and non-structural proteins at mRNA and protein level, using a panel of monoclonal antibodies and in situ RNA hybridization in lymphoid cell lines acutely and chronically infected with HIV-1. Our results indicate that in acute HIV infection four distinct phases, namely latent, regulatory, productive and cytopathic, can be demonstrated with clear qualitative and quantitative differences in the expression of viral proteins. Furthermore, we demonstrate that Nef and Tat proteins may shuttle between the nucleus and cytoplasm depending on the stage of the infectious cycle, and

that Rev is found mainly in the cytoplasm when an antibody that recognized the RRE binding region of the Rev protein is used.

Materials and methods

Virus and cell lines

HIV-1 isolates IIIB, MN and RF and the lymphoid cell line H9 were obtained from Dr. R. C. Gallo, LTCB, NCI, Bethesda, U.S.A. The MT4 cell line, abortively infected with HTLV-I [20] was a gift from Dr. G. Biberfeld, SBL, Stockholm, Sweden.

Infection experiments

The cells to be infected (107 cells) were mixed with 200 ml of fresh tissue culture medium from chronically HIV-infected H9 cells that had been supplemented 48 h earlier with uninfected H9 cells. The target cells were then incubated at 37 °C for 1 h and washed once with culture medium. An aliquot of the inoculum was collected simultaneously and MOI was determined with the Reed and Muench method [44] by titrating the virus-induced cytopathicity on MT4 cells as described earlier [31]. With this standardized procedure a MOI of 1–2 was regularly achieved.

Monoclonal antibodies

Monoclonal antibodies (MAbs) against HIV-1 Nef, Rev and Tat proteins were produced by immunizing Balb/C mice with respective recombinant proteins and by subsequently fusing the immune spleen cells with the HAT-sensitive myeloma cell line SP-2, as previously described [43].

The epitope specificity of the monoclonal antibodies was identified with the aid of synthetic peptides, fixed on solid phase (Pepscan), as previously described [53] and the epitopes recognized by each antibody are given in Table 1. The antibodies were purified from hybridoma tissue culture fluid by 50% ammonium sulphate precipitation, dissolved in PBS at a concentration of 500 µg/ml and used in dilution 1:100–1:200. Anti-gp160 monoclonal in ascites fluid (a gift from Dr. P. Durda, Du Pont) was used in dilution 1:500–1:1000.

Probes used for mRNA in situ hybridization

A plasmid containing fragment of HIV-1 (clone BH10) gag-pol gene (pGEM4GAGPOL) was obtained from Lofstrand Labs Limited, Gaithersburg, MD, U.S.A. The HIV-1 nef gene from HIV_{BRU} clone (nucleotides 8390–9010, Human Retrovirus and AIDS database, Los Alamos, NM, U.S.A.) [39] was cloned as a PCR-amplified fragment into pGEM3 vector (Promega Corporation, Madison, WI, U.S.A.) to yield plasmid pGEM3NEF. Both plasmids were linearized, and ^{35}S-UTP-labelled RNA probes were prepared according to the RIBOPROBE Gemini System protocol (Promega Corporation, Madison, WI, U.S.A.) The probes were designated as GAG/POL and NEF, respectively. Sense transcripts were used as negative control probes.

In interpretation of the in situ hybridization results, the fact that nef-specific sequences are present in all mRNA species and gag-pol specific sequences only in the full-length mRNAs was taken into account.

Table 1. Monoclonal antibodies used for the immunohistochemical demonstration of HIV-1 Nef, Rev and Tat proteins

Clone	Isotype	Epitope	Dilution
Anti Nef			
3F2	IgG1	31–40	1:100
3D12	IgG1	31–50	1:100
2F2	IgG1	151–170	1:100
3E6	IgG1	170–190	1:100
2H12	IgG1	170–190	1:100
Anti Rev			
4.0	IgG1	33–48	1:100
10.1	IgG1	33–48	1:100
Anti Tat			
1.2	IgG2a	1–16	1:200
Anti gp160[a]	IgG	NA	1:1000

[a]Ascites fluid. A gift from Dr. P. Durda, Du Pont
NA Not available

Immunohistochemistry and in situ hybridization

Cytospin cell preparates of the cultured cells were fixed in 4% paraformaldehyde for 1 minute, immersed and stored in 70% EtOH prepared in DEPC-supplemented water. Immunohistochemical staining, using the above monoclonal antibodies (Table 1), was performed as described in detail previously [46]. The bound antibody was visualized with avidin-biotin-peroxidase (Vectastain, Burlingame, CA, U.S.A.), and diaminobenzidine (DAB, 1 mg/ml; Sigma, St. Louis, MO, U.S.A.).

Each individual monoclonal antibody against Nef and Rev was tested separately to rule out the possibility that the observed findings would be due to nonspecific reactivity with one antibody, but no difference to the pattern observed with the antibody mixtures was seen. Noninfected H9 and MT4 cells were always negative.

The combined immunoperoxidase and mRNA in situ hybridization was performed as follows on precleaned cytospin slides. The immunohistochemical staining was performed first, strictly under RNase-free conditions, as described above. After the colourigenic reaction, the sections were treated with 4% paraformaldehyde, dehydrated in 70% DEPC-EtOH, washed in 2 × SSC (0.3 M NaCl/0.03 M Na-citrate), and acetylated with 0.1 M triethanolamine and acetic anhydride (pH 8.0). Thereafter, the slides were immersed in 0.1 M glycine/0.1 M Tris-HCl for 30 min, washed with 2 × SSC and dehydrated. The hybridization was carried out with ^{35}S-labeled probes at 50 °C for 3 h under rubber cement-sealed coverslips as described previously [21]. The hybridization solution contained 1×10^6 dpm of labeled probe/μl, 2 mg/ml BSA, 10 mM DTT, 50% formamide, 1 mg/ml yeast tRNA, 1 mg/ml sheared salmon sperm DNA, in 2 × SSC. The hybridized slides were rinsed in 50% formamide in 4 × SSC at 52 °C, in 2 × SSC, dehydrated and treated with RNse T_1 (1 μg/ml; Boehringer Mannheim) and RNse A (100 μg/ml; Sigma) in 2 × SSC for 30 min to remove unbound RNA, washed in 2 × SSC, dehydrated in graded alcohol series, and autoradiographed for 5–7 days using LM-1 nuclear track emulsion (Amersham,

U.K.). The slides were developed and counterstained with methyl green. The criterion for a positive hybridization reaction was at least 10 silver grains per cell and no background reaction. The sense probes generally gave no grains or rarely 1–2 grains/cell.

The same in situ hybridization protocol, starting from the acetylation step, was used when a set of slides were only hybridized with the nef probe. In all hybridization experiments, cytospin slides from the same aliquot of chronically infected H9 cells were used as positive controls.

Results

HIV expression kinetics in acutely infected MT4, H9 and A3.01 cells

In order to study the expression of HIV mRNAs and proteins through the various phases of HIV infectious cycles, MT4, H9 and A3.01 cells were infected with a large dose (1–2 MOI/cell) of HIV-1 IIIB, RF or MN isolates. In MT4 cell line with an integrated HTLV-I genobe, these HIV-1 isolates are highly cytopathic leading to cell depth mainly through syncytia formation. Cytopathic changes, both in form of syncytia formation or single cell death, are also seen with these isolates in the H9 cell cultures.

At 3 h after the infection, neither mRNA nor regulatory proteins could be detected (Table 2). However, input virus could be demonstrated, with antibody to gp160, mainly at the plasma membranes up to about 6 h post infection. At 6 h, 40% of the cells expressed spliced mRNA species (positive hybridization signal with the NEF probe) while no full-length message could be detected (no hybridization signals with the GAG/POL probe) (Table 2). At this time point, approximately 30–60% of cells expressed Rev and Tat proteins and somewhat fewer cells expressed Nef protein. The localization was exclusively cytoplasmic for Rev and Nef (Figs. 1A, 1B), and less than 1% of the Tat-positive cells showed nuclear staining for Tat. The cells expressing Rev protein were most frequent but the staining was of low intensity.

During the course of the infection, an increasing number of cells with intense nuclear staining for Tat appeared (Figs. 1C, 1D, Table 2). However, even at 24 h post infection, a few cells still expressed Tat in the cytoplasm. These cells were almost always negative for full-length HIV mRNA expression. Thus, by 24 h, three patterns of Tat expression were observed: firstly, cells with nuclear Tat protein and full-length mRNA; secondly, cells with cytoplasmic Tat protein and no full-length mRNA expression, and thirdly, a few cells with both cytoplasmic Tat protein and full-length mRNA expression (Table 3). In most multinucleated gaint cells, in addition to strong nuclear Tat positivity, also cytoplasmic staining was seen. In mitotic cells, Tat immunoreactivity was sometimes seen as dots along the chromosomes (Fig. 1D).

At 24 h, the Nef protein was still expressed mainly in the cytoplasm with a characteristic polar pattern, indicating localization in the Golgi complex, as demonstrated earlier [43] (Fig. 1E). A strong reaction for Nef protein was typically seen in the pseudopodes of fusing cells and in the cytoplasm of syncytia-forming cells. A substantial number (in average 10%) of cells showed

Table 2. Expression of full-length and spliced mRNAs and Tat, Rev, Nef and gp160 proteins in acutely infected MT-4 and A3.01 and in chronically infected H9 cells

Cell line	Time (hrs)	mRNA in situ		Tat protein		Rev protein		Nef protein		gp160 protein		
		gag-pol[a]	nef	cytopl.[b]	nucl.[b]	cytopl.	nucl.	cytopl.	nucl	cell member.	cytopl. nucl.	member.[b]
MT-4	3	–[c]	N.T.[d]	–	–	–	–	–	–	++	–	–
and H9	6	–	+	++	–	++	–	+	–	(+)	–	–
	9–12	+	+	++	+	++	–	+++	–	–	–	–
	24	++	++	+	++	+++	–	+++	+	–	++	+
	48	++	++	+	++	+	–	+++	–	–	+	+
	56	+++	N.T.	++	+	+	–	+++	–	N.T.	N.T.	N.T.
A3.01	6	N.T.	++	+	–	+	–	+	–	++	–	–
	13–16	–	++	++	–	++	–	++	(+)	–	–	–
	24	N.T.	++	+	++	++	–	+++	+	–	+++	++
	36–48	N.T.	+++	–	++	++	–	+++	+	–	+++	++
	60	N.T.	++	+	++	+	–	+	–	N.T.	N.T.	N.T.
H9 Chronic infection		+++	+++	+	++	+++	–	+++	+	–	+++	+

[a]Specificity of the probes used for in situ hybridization: gag-pol detects only full length mRNA; nef detects all mRNAs

[b]*cytopl.* Cytoplasmic immunostaining, *nucl.* nuclear immunostaining, *cell membr.* staining along cell membrane, *nucl. membr.* staining along nuclear membrane

[c]The proportion of cells positive in in situ hybridization or in immunostaining for the indicated protein is depicted as follows: – denotes all cells are negative, (+) denotes borderline reactivity, + denotes less than 30% of cells are positive, ++ denotes 30–60% of cells are positive, and +++ denotes that >60% of cells are positive

[d]*N.T.* Not tested

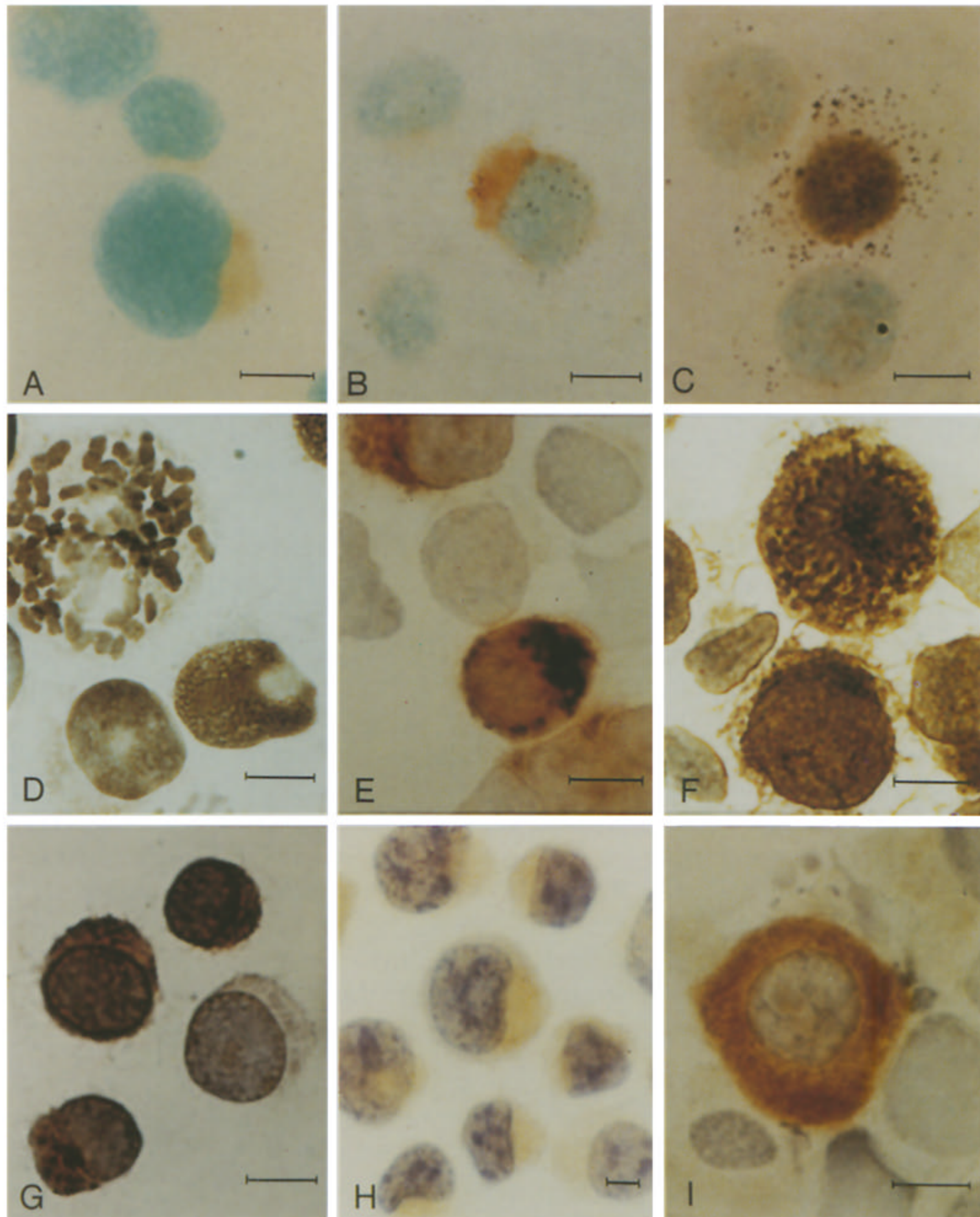

Fig. 1. Expression of HIV regulatory proteins Nef, Rev and Tat and mRNA detected with gag-pol or with nef probes in MT4 cells at different time points after the infection. The immunostained proteins are visualized by the reddish-brown colour product and the black silver grains depict the hybridized mRNA. **A** Appearance of Rev protein in MT4 cell cytoplasm and the absence of silver grains indicative of gag-pol mRNA expression at 5 h postinfection. **B** Expression of cytoplasmic Tat protein and nef mRNA (black silver grains) at 6 h post infection. **C** MT4 cell at 24 h post infection expressing nuclear Tat protein and abundant mRNA detected with the *nef* probe. **D** Nuclear and chromosomal location of Tat at 39 h. **E** Typical localization of Nef protein 21 h post infection. **F** Nuclear, cytoplasmic and chromosomal location of Nef protein 24 h post infection. **G** Nuclear localization of Tat protein at 36 h. **H** Rev protein located in the cell cytoplasm 28 h post infection. **I** MT4 cell at 58 h with strong cytoplasmic staining for Rev protein

Table 3. Interrelation of nef mRNA and TAT protein expression in lymphoid cell lines infected with HIV-1

Target cell and duration of HIV infection	Percentage of cells expressing nef mRNA/Tat protein			
	+/+	+/−	−/+	−/−
H9, chronic	46	20	11	23
MT4, 6 h	24	23	14	40
MT4, 24 h	30	8	46	16
A3.01, 6 h	7	28	2	63
A3.01, 24 h	22	18	15	45

Nef localization also in the nucleus at this stage. In analogy to Tat reactivity, some mitotic cells showed Nef immunoreactivity as dots along the chromosomes (Fig. 1F). At this time point, the subcellular localization of Nef protein resembled that of Tat (Fig. 1G) but also the newly synthesized gp160. Gp160 protein, however, was usually more abundantly localized at the nuclear and cytoplasmic cell membranes.

In contrast to Nef and Tat, no nuclear Rev protein expression was observed although the number of Rev-expressing cells increased at 24 h (Fig. 1H). At 48 h, the number of Rev-expressing cells dramatically decreased but a very intense cytoplasmic staining was detected in the remaining 10–20% of Rev-positive cells (Fig. 1I). The Rev-positive cells were mostly negative for HIV full-length mRNA.

At 56 h post infection and later, the majority of the MT4 cells tended to die or were undergoing degenerative changes. At this stage, Tat again localized almost exclusively in the cytoplasm and only a few cells with nuclear staining were observed (Table 2).

Expression of HIV in chronically infected H9 cells

In H9 cells chronically infected with HIV-I IIIB, MN or RE, 80–95% of cells gave a strong positive signal (several hundred grains/cell) with the two probes used for in situ hybridization, indicating the simultaneous expression of full-length and spliced mRNAs. From 50 to 90% of cells were positive for gp160, Nef and Rev proteins, and 20–60% for the Tat-protein (Table 2). Tat was localized almost exclusively in the nuclei although in some cells (less than 5%) Tat staining was seen in the cytoplasm. Nef was almost exclusively cytoplasmic but in a few cells (less than 5%) both cytoplasmic and nuclear staining for Nef could be demonstrated. Rev protein was detected in most cells and the localization was, without exception, in the cytoplasm. Cytoplasmic Rev reactivity was diffuse and thus contrasted to the polar pattern seen with the anti-Nef antibodies.

In experiments where immunohistochemistry combined to in situ RNA hybridization was applied to chronically infected H9 cultures, most cells expressing Nef and Tat proteins also expressed full-length mRNAs. For example, 88% of cells in chronically infected H9 cultures were simultaneously positive for both Nef protein and for the GAG/POL mRNA while only 2% of the Nef-positive cells were negative for this probe. On the other hand, 6% of the cells that were negative for Nef protein, expressed mRNA for the structural proteins.

Discussion

In the present work, we have studied the cellular localization, expression kinetics and mutual relationship of HIV regulatory and structural proteins as well as the corresponding mRNAs in acutely and chronically infected lymphoid cell lines. Our results indicate that the in vitro infectious cycle of HIV can be divided into distinct phases on the basis of both quantitative and qualitative differences in the expression of viral mRNA and protein species (Table 4).

The first (latent) phase extends from zero to c. 6 h post infection. During this time period, only proteins from the incoming virus may be demonstrated by immunostaining. The viral RNA is reverse transcribed and integrated to the host cell genome [6, 27]. The second, regulatory phase (from 6 to 12 h), is characterized by the appearance of abundant amounts of spliced mRNA species and Nef, Rev and Tat proteins in the cytoplasm, while the mRNAs for Gag and Pol proteins can not be demonstrated until 9 to 12 h. Earlier work has demonstrated that the viral structural proteins are not synthesized before the corresponding non-spliced or singly spliced mRNA species are transported from the nucleus into the cytoplasm [27, 45] and that the Rev protein is necessary for this transportation [51]. Our present findings support the observation by Kim and co-workers who demonstrated, by northern hybridization, the appearance of 2 kb mRNA at 12 h and full-length genomic RNA only after 20 h [27]. A similar, although slower (starting at 24 h) ordered appearance

Table 4. Summary of HIV-1 expression kinetics in acutely infected lymphoid cell lines

Stage	Time (h)	Protein expression							mRNA expression	
		Tat		Nef		Rev		gp160	Nef	gag/pol
		Cyt	Nucl	Cyt	Nucl	Cyt	Nucl			
Latent	0–6	−	−	−	−	−	−	+ (incoming virus)	−	−
Regulatory	6–9(12)	++	(+)	++	−	++	−	−	++	(+)
Productive	12–48	+	++	+++	+	+++	−	++	++	+++
Cytopathic	>48	++	−	+++	−	+	−	++	−	+

of HIV transcripts has been shown to occur in macrophages [37]. Thus, not only the transport from nucleus and translation of mRNA, but also the transcription schedule of the viral genome is controlled by regulatory mechanisms. Characteristically, during the early regulatory phase of HIV infection, Nef, Rev and Tat are all localized in the cytoplasm. This fits with the concept that the structural proteins are not expressed before the regulatory proteins enter the nucleus. The finding that Nef is the most abundantly expressed protein at this stage would suggest that Nef has some regulatory function.

In the third, productive phase, extending from 12 to 48 h, all viral mRNAs as well as structural and regulatory proteins are synthesized. At this stage, production of infectious viral particles has already started and some free infectious virus or p24 antigen can be demonstrated at 24 h in the tissue culture fluid (data not shown). Finally, in cell cultures subject to the cytopathic effect of HIV, the last phase, starting at 48–56 h post infection is characterized by pycnotic nuclear changes and cell depth.

Earlier work has shown that Tat and Rev are nuclear proteins so that Tat resides in the perinucleolar region and Rev in the nucleous [11, 22, 35, 47], but that Nef is localized in the cytoplasm [13]. We now show, firstly, that all three regulatory proteins (Nef, Rev, Tat) are localized in the cytoplasm, and secondly, that later Nef and Tat translocate into the nucleus and, thirdly, that Rev is predominantly seen in the cytoplasm in natural infection. For Rev localization, we could use only antibodies that recognized the RRE binding site of the Rev protein and thus presumably mainly reacted with "free" protein, not bound to the full length and singly-spliced mRNA species in the nucleus. The findings concerning Nef extend our earlier observation of its occasional nuclear localization in the chronically infected H9 cell line. Localization of Nef both in the cytoplasm and in the nucleus has recently been confirmed by other groups using either light microscopy [26, 30] or immunoelectron microscopy [38]. The nuclear localization of Nef seems to be dependent on the infectious cycle and coincides with the peak of viral production. Nef occurs in two isomorphic forms [43], possibly due to an alternative start of the translation from the 10th and/or 20th amino acid (Met) and thus leading to the shorter forms. It is thus possible that the variable subcellular localization of Nef protein is regulated by the choice of initiation codon, as has been demonstrated with other nuclear proteins [1, 5]. Alternatively, the nuclearly localized Nef protein may be a non-myristoylated variant, carrying alanine instead of glycine at position 2 [30, 54]. It was recently suggested [38] that a 16-mere amino acid sequence close to the N-terminus of Nef would be responsible for its nucleoplasmic localization. If the two isomorphs of Nef were in fact the result of variation in the usage of initiation codon, then the longer Nef protein (27 kDa) would be localized in the nucleus. This possibility could not be confirmed in the present work, as none of the anti-Nef antibodies used reacted with the first 20 amino acids of the Nef protein. Taken together, our findings suggest that at least one isomorph of Nef may act as a nuclear regulatory factor, although not by binding to DNA itself but possibly through protein-protein interactions

with cellular factors. Samuel and co-workers have shown that HIV Nef proteins bear a leucine repeat structure resembling the leucine zipper motif found a transcription factors [48], which might thus allow heterodimerization of Nef with transcription factors in the cytoplasm and subsequent transport into nucleus.

The present work describes expriments performed with only a few HIV-1 isolates, all adapted to growth in established cell lines. Also, only three target cell lines were tested. It is likely, however, that the expression kinetics of HIV-1 mRNA and protein species would show quantitative and qualitative differences that are related to the phenotype of the viral isolate and/or host cell. Such studies, using peripheral blood lymphocytes and monocytes as well as viral isolates with slow/low or rapid/high phenotype are now under way.

The demonstration of a distinct phase of HIV replication cycle, with the expression of regulatory proteins Rev, Tat and Nef while the structural proteins are not yet expressed, bears relevance to HIV vaccine development. So far, the main efforts in HIV vaccine development have focused in the envelope-based preparations which would mainly raise a humoral immune response operating through neutralizing antibodies. An alternative mechanism for protection would be a strong cytotoxic T lymphocyte (CTL) response capable of destroying virus-infected cells. If the CTL response could be targeted towards the early expressed regulatory proteins, infected cells would be destroyed before the release of mature infectious viral particles.

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