Enhancement of nucleocytoplasmic export of HTLV-1 Rex mRNA through *cis* and *trans* interactions of the mRNA with the complex of Rex protein and Rex-responsive element

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Abstract p27 rex of HTLV-1 promotes nucleocytoplasmic export of viral mRNAs through binding of the Rex-response element (RexRE) present at the 3′ end of the viral transcripts in *cis* with respect to the ORFs of the viral mRNAs. We have found that expression of the RexRE in *trans*, as a separate RNA, still allows Rex protein to promote export of viral mRNAs lacking the RexRE. The data suggest the formation of a ternary complex between Rex protein, RexRE and upstream elements of viral mRNA and hence the existence of secondary sites of interaction between Rex protein and viral RNAs.

Key words: mRNA export; HTLV-1; Retrovirus; p27rex; Rex-responsive element; Fluorescence in situ hybridisation

1. Introduction

Human T-cell leukaemic virus type 1 (HTLV-1) is a retrovirus trophic for $CD4^+$ T-cells associated with an aggressive form of leukaemia, adult T-cell leukaemia [1,2], and with a neurological disorder, tropical spastic paraparesis, also due to infection of T-cells [3]. In order to co-ordinate the regulation of the structural and viral proteins necessary for viral replication a nuclear protein, Rex, encoded in the viral genome, makes efficient use of variably spliced reading frames in the viral genome (reviewed in [4]). In the presence of Rex incompletely spliced transcripts of viral RNA are expressed in the cytoplasm, allowing expression of proteins from the unspliced *gag-pol* transcript and the singly spliced *env* transcript. In the absence of Rex the incompletely spliced transcripts remain sequestered in the nucleus to be degraded or for completion of splicing [5,6]. Completely spliced transcripts encode the regulatory proteins of the virus, Tax and Rex [5]. Rex works by binding directly to a highly structured element of RNA of about 250 bases present in the viral transcripts, the Rex-responsive element (RexRE) [7–9]. Details of the mechanism of action of Rex, and its analogue Rev from human immunodeficiency virus (HIV) type 1, are becoming apparent. Despite their lack of sequence homology both proteins contain two corresponding functional domains. The N-terminal domain is rich in basic amino acid residues and serves as a nuclear localisation signal, which also has nucleolar localising capability [10]. In addition the same domain is the site for multimerisation [11] and for binding to the responsive element on the viral RNA [8,12]. A C-terminal domain encodes a leucine-rich effector domain which includes a recently characterised nuclear export signal [13,14]. The combination of nuclear import and export signals accounts for the demonstrated ability of Rev to shuttle between the nucleus and cytoplasm [15,16].

Two models have been proposed to account for the retention of unspliced viral mRNAs in the nucleus and for the relief of the retention by Rex or Rev. In one the splicing signals of the introns of the larger transcripts of viral RNA are inefficient, and in the absence of Rex or Rev the unspliced RNAs are retained in the nucleus bound to spliceosome complexes [17]. Rex or Rev can partially dissociate the spliceosome to a smaller commitment complex comprising the mRNA, U1 snRNP and possibly U2 snRNP, having displaced the U4/U6.U5 tri-snRNP complex [18]. Cellular factors would then complete the dissociation of the mRNA from splicing snRNPs and allow Rex or Rev to carry the RNA out of the nucleus. In the second model regulatory sequences in the RNAs outside the splice sites are responsible for the retention of the RNAs in the nucleus [19]. Such sequences have been mapped to several locations on HIV [19–23]. Rex or Rev complexed with their responsive elements would overcome the retention and drag the freed mRNA out of the nucleus.

A question we have been concerned with involves the nucleolar localisation sequences present in both Rev and Rex which coincide with the RNA binding regions. The nucleolar localisation signal (NOS) of Rex is contained in the N-terminal 20 aa and can direct nucleolar localisation of chimeras of proteins normally expressed in the cytosol [10]. A functional NOS in Rex has been shown to be critical for Rex activity – point mutations which abrogate the nucleolar localising capacity of the protein also inhibit completely the ability of Rex to promote transport of unspliced forms of viral RNA out of the nucleus [24]. Rex has also been found to stabilise the mRNA of the Tac subunit of the receptor for IL-2 [25], and an intact NOS is required for the activity [26]. Mutation of the NOS can abrogate both RNA binding and nucleolar localisation and it is not clear from these data whether a lack of Rex in the nucleoli is part of the reason for the loss of the functioning of Rex in vivo. Although Rex is heavily expressed in nucleoli staining in the nucleoplasm is also evident [10] and detailed immunohistochemical analysis of Rev localisation reveals a variety of staining patterns in the nucleolus, nucleoplasm and cytoplasm [27]. Chimeras of Rev activation domain and transcription factor DNA binding domains indicate that the effector domain of Rev can work outside nucleoli in the nucleoplasm [28]. In an effort to determine whether the site of

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Abbreviations: FITC, fluorescein isothiocyanate; HIV, human immunodeficiency virus; HTLV-1, human T-cell leukaemic virus type 1; NOS, nucleolar localisation signal; RexRE, Rex-responsive element

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interaction of Rex protein and viral mRNA is in the nucleolus or in the nucleoplasm, we analysed the intracellular distribution of HTLV-1 viral RNAs by fluorescence in situ hybridisation. We could not detect viral RNAs in the nucleoli but found a novel type of interaction between the Rex/RexRE complex and upstream elements of the viral RNA.

2. Material and methods

2.1. Cell culture and transfection

All experiments were carried out with COS7 cells (ECACC) cultured in DMEM with 10% fetal calf serum, 2 mM glutamine and 25 μg/ml gentamycin. Cells were seeded on 22×22 mm coverslips 1 day prior to transfection. A 10 cm dish was transfected with 10 μg of plasmid using DEAE-dextran [29]. Cells were cultured for 3 days after transfection before in situ hybridisation.

2.2. Expression plasmids

All expression plasmids were constructed in pcDNA1 (Invitrogen) which contains a promoter-enhancer from cytomegalovirus and uses splicing and polyadenylation signals from the early SV40 genes. The organisation of the plasmids and probes used in the study are shown in Fig. 1. The cDNA of the fully spliced transcript of HTLV-1 mRNA was subcloned into pcDNA1 to form pcDNA-RexLTR. The viral cDNA was subcloned as a *Hin*dIII fragment pKCR27x [10]. pcDNA-Rex contained the first half, a *Hin*dIII-*Sma*I fragment, of the viral cDNA used in pcDNA-RexLTR. pcDNA-LTR carries the 3′ half, the *Sma*I-*Hin*dIII fragment, of the viral cDNA used. pcDNA-Rex′ and pcDNA-Rex′LTR were constructed by digesting pcDNA-Rex and pcDNA-RexLTR at the *Sph*I site, which contains the initiation codon of Rex, blunting to remove the codon and religating.

2.3. Fluorescence in situ hybridisation

The method described in White and Fu [30] was followed exactly. Briefly, mRNAs expressed from transiently expressed pcDNA constructs were detected using riboprobes labelled with digoxigenin (Boehringer Mannheim). Hybridised probe was detected with a double application of antibodies – first was the anti-digoxigenin antibody and the second antibody was labelled with FITC. Cells were counterstained in 0.05 μg/ml of propidium iodide to visualise nuclei. Cells were viewed under an Olympus OM2 fluorescence microscope and photographs taken on Ilford FP4 monochrome. Rex mRNAs expressed from transfected plasmids were detected using antisense riboprobes synthesised over the whole length of the Rex open reading frame.

3. Results

3.1. Assessment of intracellular distribution of mRNA by FISH

We were interested to establish whether Rex binds to the RexRE of cognate RNAs in nucleoli in view of the strong nucleolar localisation signal present on Rex protein [10]. We chose to locate viral RNAs expressed from segments of HTLV-1 in COS cells using the high resolution of fluorescence in situ hybridisation. We could never observe nucleolar staining of fully spliced viral mRNAs, although the technique was capable of detecting naturally occurring nucleolar RNAs such as U3 and 18S RNAs (data not shown). When assessing expression of Rex mRNA with and without the LTR present we found a clear difference in intracellular distribution. Viral RNA without the LTR was expressed well but largely retained in the nucleus, whereas RNAs containing the LTR were expressed well in the cytoplasm. To investigate further we assessed different intracellular distributions of expressed mRNAs semi-quantitatively by scoring cells according to four patterns of staining reflecting a gradation of distribution from nucleus to cytoplasm: (a) staining almost exclusively nuclear with no perinuclear signal (N, Fig. 2a); (b) more nuclear than cytoplasmic in which staining in the nucleus was strong and a lesser degree of perinuclear and or cytoplasmic staining was evident (NC, Fig. 2b); (c) more cytoplasmic than nuclear with weak nuclear staining with strong perinuclear and or cytoplasmic staining (CN, Fig. 2c); (d) almost exclusively cytoplasmic with little or no nuclear staining and a strong signal in the cytoplasm (C, Fig. 2d). We scored over 100 cells for each type of plasmid transfected, in at least three experiments per plasmid, and represent the data normalised as percentage distributions between the four categories.

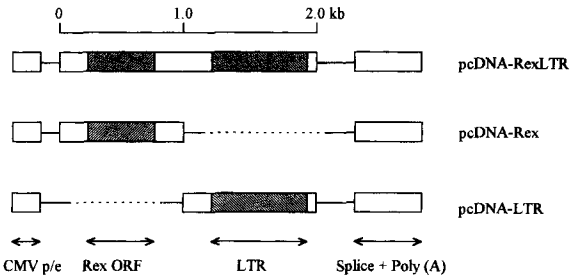

Fig. 1. Plasmid constructs used in the study.

3.2. Without the LTR Rex mRNA is expressed well but strongly retained in the nucleus

Rex mRNA expressed from pcDNA-Rex, which contains Rex cDNA without the viral LTR (Fig. 1), was found to be expressed predominantly in the nucleus with some cytoplasmic staining (Fig. 3a). In total about 70% of the cells scored had Rex mRNA exclusively or mainly in the nucleus. The presence of strong staining in the nucleus suggests that the cDNA is transcribed efficiently but the RNA is exported poorly from the nucleus. mRNAs which are exported efficiently from the nucleus usually show little staining in the nucleus and strong staining in the cytoplasm (Fig. 2d) suggesting that the Rex mRNA is retained in the nucleus. The small amount of cytoplasmic signal may be due to saturation of nuclear binding sites allowing excess mRNA to be exported [31].

3.3. Co-expression of LTR RNA either in cis or in trans to Rex mRNA allows full cytoplasmic expression of Rex mRNA

Rex mRNA expressed from pcDNA-Rex was expressed in the nucleus well but exported to the cytoplasm poorly. We reasoned that addition of the LTR to the Rex cDNA should promote cytoplasmic expression of Rex mRNA, through the action of Rex protein on the RexRE in the LTR. Although most of the Rex mRNA was retained in the nucleus some cytoplasmic staining is evident which should lead to synthesis of the Rex protein required for promoting nucleocytoplasmic export of the viral mRNA. We transfected COS cells with pcDNA-RexLTR and assessed distribution of Rex mRNA (Figs. 2d and 3, middle graph). Expression of mRNA was high and export to the cytoplasm efficient. About 80% of the cells scored had Rex mRNA predominantly in the cytoplasm with very little nuclear staining, compared with about 5% in cells transfected with pcDNA-Rex (Fig. 3a,b). In a serendipitous experiment we assessed expression of Rex mRNA when LTR RNA was co-expressed from a separate plasmid in *trans*. Surprisingly the Rex mRNA was found to be

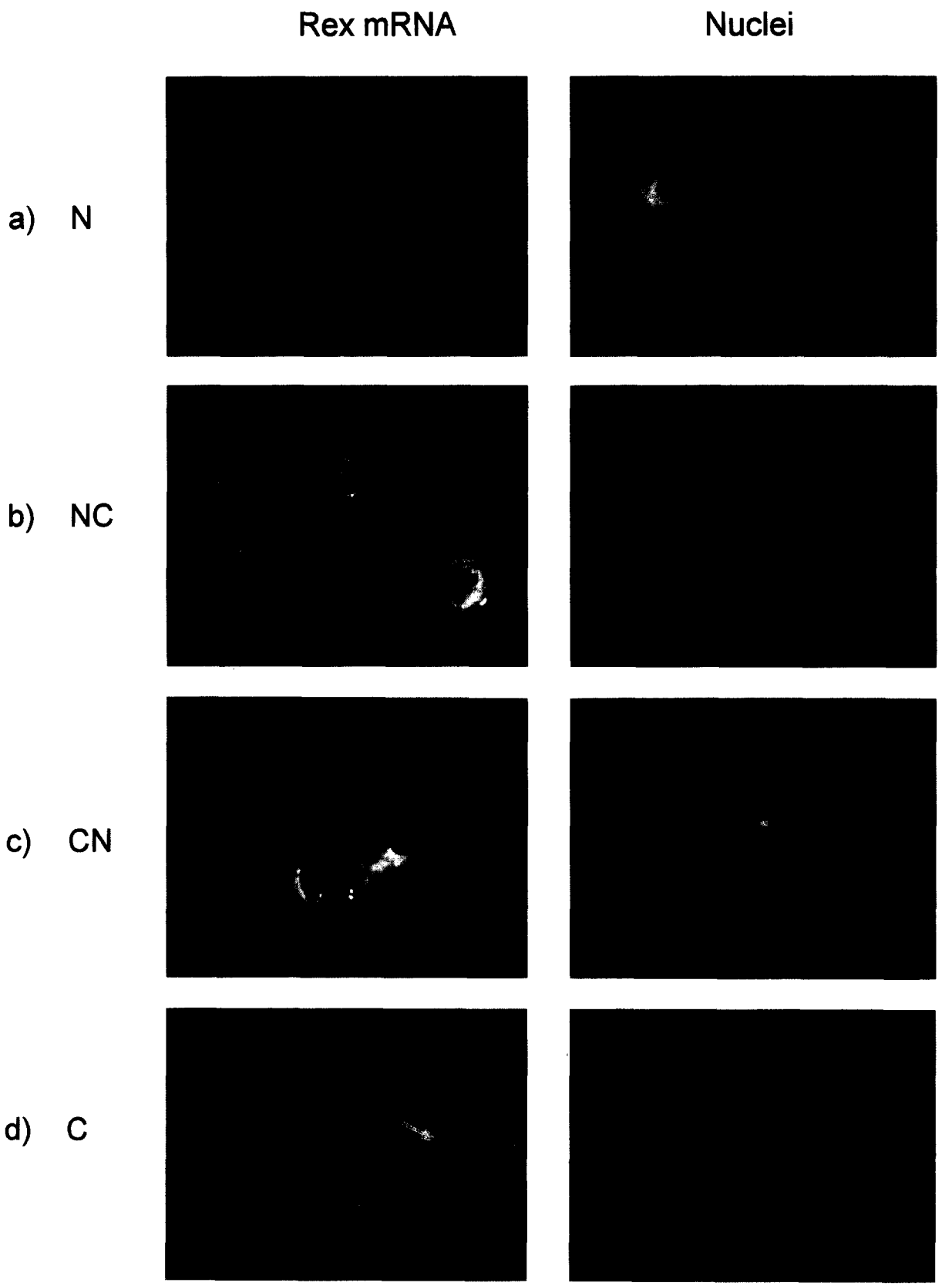

Fig. 2. Intracellular distributions of Rex mRNA detected by fluorescence in situ hybridisation. Examples of the four categories of distribution are shown. (a) Exclusively nuclear mRNA, expressed from pcDNA-Rex'. (b) Predominantly nuclear mRNA, expressed from pcDNA-Rex. (c) Mainly cytoplasmic mRNA but with some staining in the nucleus, expressed from pcDNA-Rex co-transfected with pcDNA-LTR. (d) Almost exclusive staining in the cytoplasm, indicated by the white arrow, expressed from pcDNA-RexLTR. Another cell with CN type of distribution is also evident. (a–c) are 400× magnification and (d) is 200×. Cells were counterstained with propidium iodide to visualise nuclei.

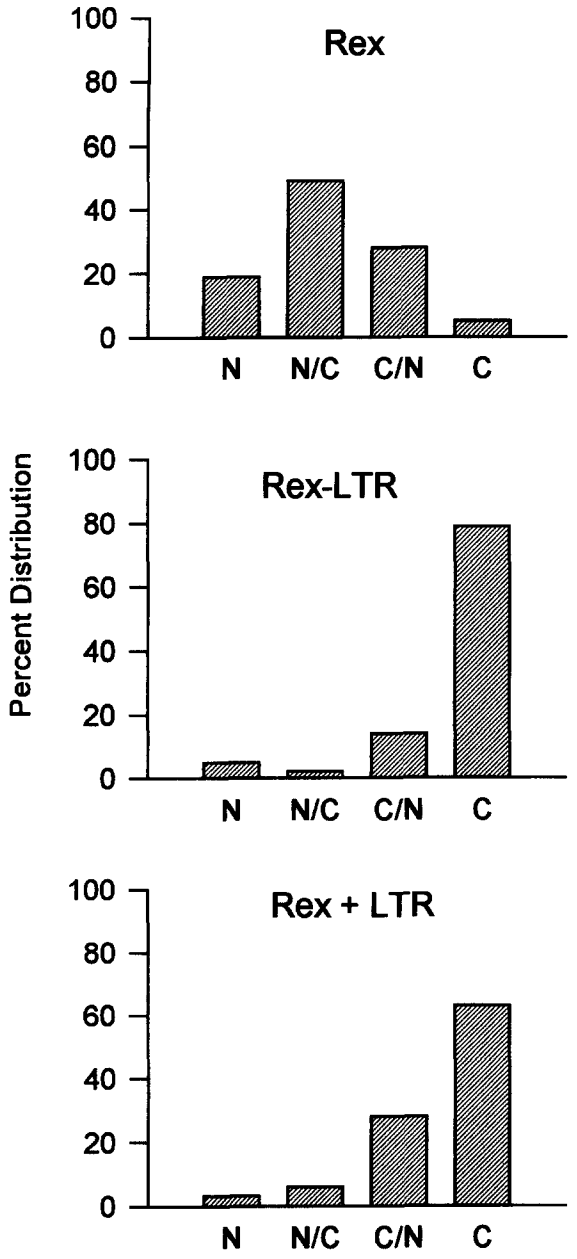

Fig. 3. Comparison of distribution of Rex mRNA in cells transfected with pcDNA-Rex, pcDNA-RexLTR or pcDNA-Rex+ pcDNA-LTR. Data are from over 100 cells per plasmid and normalised as percentages.

exported efficiently to the cytoplasm to the same extent as when the RexRE is present in *cis* on the same RNA. 90% of the cells scored expressed Rex mRNA predominantly in the cytoplasm and very little nuclear localisation was evident (Fig. 3c). The presence of the LTR in *cis* on the Rex mRNA allows Rex protein to bind to the RNA and promotes export of the RNA. The fact that the LTR RNA expressed separately from Rex mRNA could also enhance export of the Rex mRNA suggested a novel interaction between Rex protein and cognate RNA involving a ternary complex of Rex protein, the RexRE and upstream elements of viral RNA.

3.4. Functional Rex protein is required for enhanced export of Rex mRNA induced by LTR DNA in cis or trans

We used a mutant of Rex to confirm that functional Rex protein was required for the enhanced export of Rex mRNA. Rex′ contained a deletion of the initiation codon. We found that Rex mRNA expressed from Rex′ was expressed mainly in the nucleus, with small amounts of cytoplasmic staining, in a pattern very similar to unmutated Rex mRNA (Fig. 4 top graph; compare with Fig. 3 top graph). The distribution was not affected by the presence of the LTR in *cis* or in *trans* (Fig. 4 middle and lower graphs), confirming that Rex protein is necessary for the enhanced export of viral mRNA stimulated by the LTR RNA. The data also show that the LTR RNA does not possess export promoting activity.

4. Discussion

Three species of mRNA are expressed from a single tran-

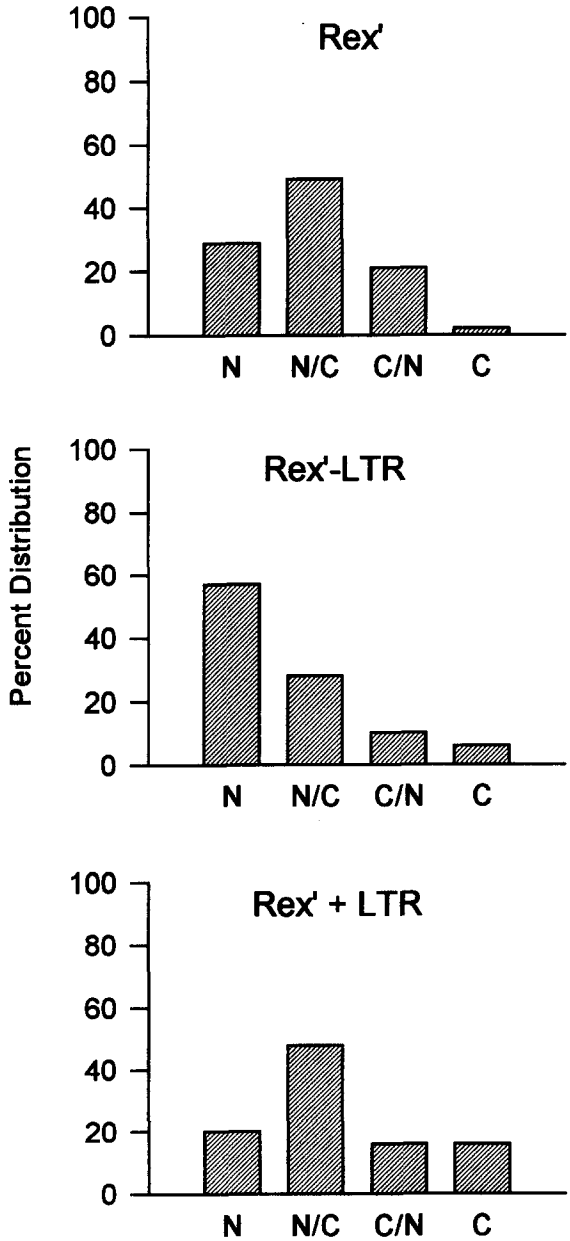

Fig. 4. Comparison of distribution of Rex mRNA in cells transfected with pcDNA-Rex′, pcDNA-Rex′LTR or pcDNA-Rex′+ pcDNA-LTR. Data are from over 100 cells per plasmid and normalised as percentages.

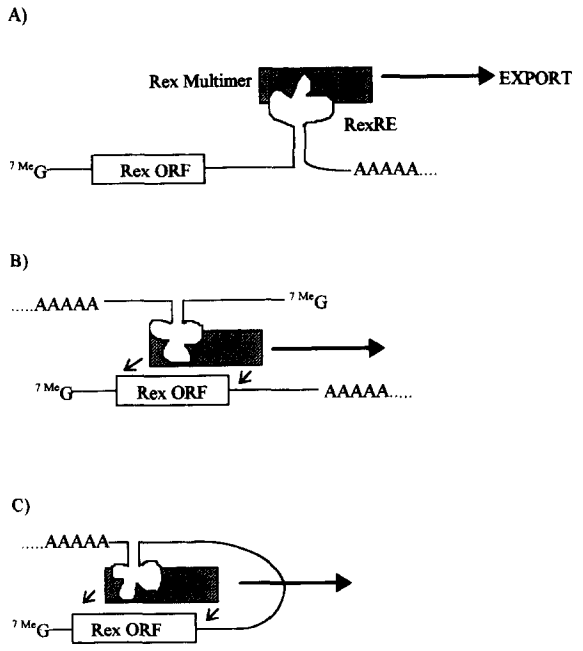

Fig. 5. Models of interaction between Rex protein and cognate viral mRNAs. (A) Simple model in which Rex binds to the RexRE, multimerises and drags viral RNA out of the nucleus. In this model Rex acts simply as a link between the RexRE on viral RNA and cellular export factors. (B) Ternary complex envisaged to form between Rex protein, the RexRE and viral mRNA expressed separately, in *trans*. The viral mRNA is co-transported by the binding of the Rex protein-RexRE complex. (C) 'Ternary complex' model, taking into account the data from the present study. See text for discussion.

scription site on HTLV-1 progenomic DNA, two of which contain introns and require the activity of Rex protein, encoded in the fully spliced transcript, for expression in the cytoplasm [5]. Promotion of expression of incompletely spliced transcripts in the cytoplasm may involve two activities of Rex. One, not yet shown directly but inferred from studies on the HIV equivalent Rev [15,16], is that Rex promotes export of viral RNAs by binding to the RexRE on the RNA and dragging the RNA out of the nucleus. In this respect a nuclear export signal has been identified on Rex [13]. The second activity would involve the ability of Rex to stabilise the incompletely spliced viral transcripts, containing one or two introns, so that detectable levels are present in the nucleus - in the absence of Rex these transcripts are almost absent [5]. In order for Rex to transport the incompletely spliced or unspliced transcript out of the nucleus Rex must overcome interactions between the viral transcripts and other nuclear factors which either retain the transcripts in the nucleus or divert them into splicing pathways. Such interactions may also involve the *cis*-repressive sequences similar to those identified in HIV which serve to down-regulate cytoplasmic expression of viral RNAs [19–23]. Thus there is scope for a role for Rex in having secondary interactions with elements of viral RNA upstream from the RexRE.

We have shown that Rex does indeed manifest binding to secondary sites of viral RNA and the data suggest that Rex does so only when bound to the RexRE. Importantly in our expression system the mRNA of Rex is relatively poorly exported, despite the presence of splicing signals in the expression vector. In general we have found the splicing signals to be important for efficient cytoplasmic expression of mRNAs expressed from cDNAs in the pcDNA1-COS system (Fu, Suen and White, unpublished data). Rex mRNA is expressed well, as judged by the intensity of staining in the nuclei (Fig. 2b), but largely retained in the nucleus when LTR RNA is absent. The retention in the nucleus could be attributed to putative *cis*-repressive sequences in the viral RNA analogous to the elements characterised in HIV [19–23]. Co-expression of LTR RNA stimulated efficient cytoplasmic expression whether the RNA was present as part of the same viral transcript, in *cis*, or as a separate transcript in *trans*. Deletion of the RexRE from the LTR RNA (data not shown), or prevention of expression of Rex protein abrogated the stimulatory effect indicating that Rex bound to the RexRE is the active factor which promotes the secondary interactions with the viral RNA. A model for Rex action is illustrated in Fig. 5. In (A) Rex acts simply to promote export through binding of the RexRE. Rex forms a multimer once bound to the RexRE and the export motifs of the multimer provide the signal for nuclear export. In (B) the model is modified to account for the observed effects of expressing the LTR RNA in *trans*. Rex forms a multimer complex with the RexRE on the LTR RNA and this complex in turn binds to sites normally upstream of the RexRE, but in this experiment on a separate RNA, to form a ternary complex. The export signals of Rex allow cytoplasmic expression of the whole complex. The binding of monomeric Rex to these secondary sites is presumably quite weak, and hence not detectable by normal gel shift assays, but multimerisation of Rex would allow an accumulation of weak interactions to a stable ternary complex. (C) reflects what is envisaged to be the normal situation in which the 'ternary' complex is formed between RexRE, Rex multimers and sites upstream of the RexRE on the RNA. In this model Rex makes initial contact with high-affinity sites on the RexRE, multimerises and then forms the secondary contacts with the viral RNA. Work is currently under way to map the sites of secondary contact and establish that unspliced forms of viral RNA respond similarly to the expression in *trans* of the RexRE.

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