# Acetylation of the HIV-1 Tat protein by p300 is important for its transcriptional activity

Melanie Ott*†, Martina Schnölzer*, Jerry Garnica†, Wolfgang Fischle†, Stephane Emiliani†, Hans-Richard Rackwitz* and Eric Verdin†

**The human immunodeficiency virus 1 (HIV-1) Tat protein activates transcriptional elongation by recruiting the positive transcription elongation factor (pTEFb) complex to the TAR RNA element, which is located at the 5′ extremity of all viral transcripts [1–3]. Tat also associates *in vitro* and *in vivo* with the transcriptional coactivator p300/CBP [4–6]. This association has been proposed to recruit the histone acetyltransferase (HAT) activity of p300 to the integrated HIV-1 promoter. We have observed that the purified p300 HAT domain acetylates recombinant Tat proteins *in vitro* and that Tat is acetylated *in vivo*. The major targets of acetylation by p300 are lysine residues (Lys50 and Lys51) in the arginine-rich motif (ARM) used by Tat to bind RNA and for nuclear import. Mutation of these residues in full-length recombinant Tat blocked its acetylation *in vitro*. Furthermore, mutation of these lysine residues to arginine markedly decreased the synergistic activation of the HIV promoter by Tat and p300 or by Tat and cyclin T1. These results demonstrate that acetylation of Tat by p300/CBP is important for its transcriptional activation of the HIV promoter.**

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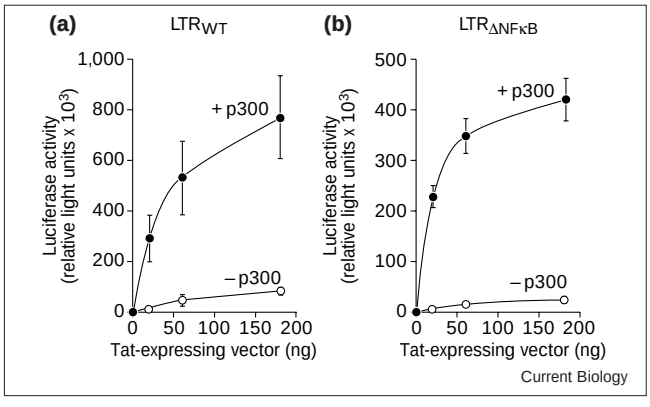
## Results and discussion

To examine the synergy between Tat and p300, we conducted transient transfection experiments with Tat and p300 expression vectors and an HIV long terminal repeat (LTR) construct driving the luciferase reporter gene. In the absence of p300, Tat increased transcription of the HIV promoter up to 42-fold (Figure 1a). In the absence of Tat, p300 had little effect on transcription (1.6-fold increase); addition of p300 to Tat caused a major superinduction of Tat transactivation (up to 256-fold). Similar results were obtained when an HIV promoter construct containing mutated binding sites for the transcription factor NFκB was used (Figure 1b). This result indicated that superinduction of Tat transactivation by p300 was dependent on an interaction between p300 and Tat rather than on the interaction between p300 and NFκB/p65. Importantly, p300 had little or no effect on the Rous sarcoma virus promoter, which controlled the expression of Tat proteins (data not shown).

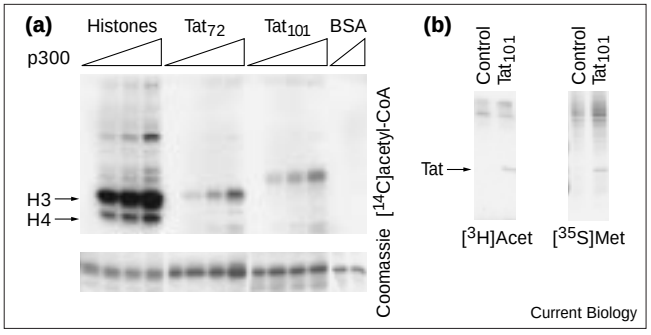
Because the superinduction mediated by p300 was restricted to the Tat-activated HIV promoter, we explored the possibility that Tat itself might be the target of p300 HAT activity. A recombinant protein corresponding to the HAT domain of p300 was expressed in *Escherichia coli* as a fusion protein with GST (GST–p300-HAT) [7] and incubated with two purified natural HIV-1 Tat isoforms of 72 and 101 amino acids ($Tat_{72}$ and $Tat_{101}$, respectively) [8] in the presence of [$^{14}$C]acetyl-coenzyme A (acetyl-CoA). Acetylation of both Tat proteins occurred in a dose-dependent manner in response to increasing amounts of GST–p300-HAT (Figure 2a). As expected, histones H3 and H4 were strongly acetylated by GST–p300-HAT and bovine serum albumin

**Figure 1**




Synergistic activation of the HIV promoter by Tat and p300. HeLa cells were cotransfected using lipofectamine (Gibco-BRL) with (a) an LTR–luciferase reporter construct (LTR$_{WT}$; 180 ng) or (b) an LTR–luciferase construct in which the two NFκB-binding sites were mutated (LTR$_{\Delta NF\kappa B}$), and increasing amounts of a Tat-expressing vector [8] (180 ng total DNA, with 20, 60 or 180 ng of Tat-expressing vector). Transfections were performed in the presence (closed circles) or absence (open circles) of a p300-expressing vector [12] (600 ng CMV-p300 or vector alone). Experiments were performed in duplicate on 80% confluent cells. Luciferase activity was measured with a standard luciferase assay 24 h after transfection [8]. The mean ± SEM of three experiments is shown.

**Figure 2**




Tat is acetylated *in vitro* and *in vivo*. (a) Acid-extracted histone proteins (~1 μg), purified recombinant $His_6$-tagged Tat proteins (1 μg, [8]), or purified BSA (1 μg, Pharmacia) were incubated in a 20 μl reaction with increasing amounts of a GST fusion protein (0, 1, 2 and 4 μl dialyzed protein) corresponding to the catalytic domain of p300 (amino acids 1195–1810) [7] in a sodium citrate buffer pH 6 [13], together with 0.1 μCi [1-$^{14}$C]acetyl-CoA for 30 min at 37°C. Reaction products were separated by SDS–PAGE and processed for autoradiography. (b) 293 cells were transfected with a vector expressing the full-length $Tat_{101}$ protein fused at its carboxyl terminus to the FLAG epitope (IBI-Kodak) or with the empty vector (control). Experiments were performed in duplicate in 10 mm dishes on 60–80% confluent cells using 60 μl lipofectamine for 6 h. Twenty-four hours after transfection, cells were pulsed for 1 h with [$^3$H]acetate (0.2 mCi/plate, ICN) in complete cell culture medium or overnight with [$^{35}$S]methionine/cysteine (0.2 mCi/ml, NEN) in methionine/cysteine-free medium and 10% dialyzed FCS. Tat was immunoprecipitated from cellular lysates with an anti-FLAG antibody (M2, Kodak) and the immunoprecipitated material analyzed by SDS–PAGE and autoradiography (see Supplementary material). Three independent [$^3$H]acetate-labeling experiments were performed with similar results.

(BSA) was not acetylated (Figure 2a). These findings demonstrate that Tat proteins are specific targets for the HAT activity of p300.

To confirm that Tat is acetylated *in vivo*, we transfected an expression vector encoding $Tat_{101}$ (FLAG epitope-tagged at the carboxyl terminus) into human 293 cells. The FLAG epitope did not interfere with the transactivating activity of Tat on the HIV promoter (data not shown). The FLAG-tagged protein could be visualized as a nuclear protein (see Supplementary material). Metabolic labeling of transfected cells with $^3$H-labeled sodium acetate for an hour and subsequent immunoprecipitation with the anti-FLAG antibody (M2 antibody, Kodak) identified a single radiolabeled protein of ~17 kDa that was absent when the empty vector was transfected (Figure 2b). The same 17 kDa band was visualized after immunoprecipitation of 35S-methionine-labeled cellular extracts with the M2 antibody (Figure 2b). Western blot analysis of the immunoprecipitated material with a polyclonal anti-FLAG antiserum (Santa Cruz Biotechnology) confirmed the identity of this protein as Tat (data not shown). To rule out acetylation of the added FLAG epitope, we observed that another Tat fusion protein tagged at the carboxyl terminus with the T7 epitope (Novagen) was also acetylated *in vivo* (data not shown). In addition, peptides corresponding to both the FLAG and to the T7 epitope were not acetylated *in vitro* by GST–p300-HAT (Figure 3b).

To map the precise site of acetylation of Tat by p300, we used a series of synthetic peptides encompassing each lysine residue of Tat (Figure 3a). Tat peptides were subjected to *in vitro* acetylation reactions with GST–p300-HAT, resolved on Tris-Tricine acrylamide gels and examined by autoradiography. A single peptide corresponding to amino acids 45–58, encompassing the ARM region of Tat, was acetylated by p300 *in vitro* (Figure 3b). Peptides corresponding to the amino-terminal tails of histones H3 and H4, but not the unrelated T7 or FLAG epitopes, were labeled by p300, confirming the specificity of the assay (Figure 3b).

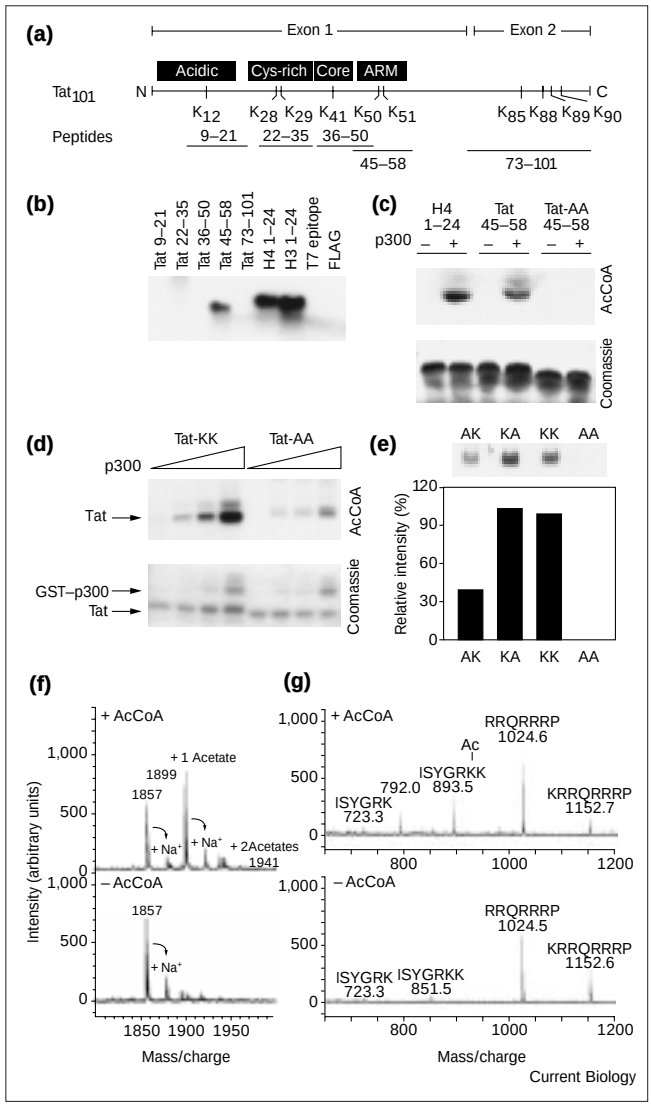
A mutant peptide in which both lysine residues (Lys50 and Lys51) were replaced with alanines was not acetylated *in vitro* (Figure 3c). Similarly, mutation of Lys50 and Lys51 in full-length recombinant Tat expressed in *E. coli* reduced p300-mediated [$^{14}$C]acetate incorporation by more than 90%, indicating that Lys50 and Lys51 are the major target of p300 acetylation in the complete Tat protein (Figure 3d).

Mass spectrometry analysis after *in vitro* acetylation of the ARM peptide (amino acids 45–58) by p300 revealed the appearance of two new products when the acetylation reaction was performed in the presence of acetyl-CoA. One major product with a molecular mass of 1,899 Da corresponded to the monoacetylated form of the peptide (42 Da larger than the unacetylated peptide) and a minor product with a molecular mass of 1,941 Da represented the diacetylated form of the peptide (+ AcCoA, Figure 3f). These new products were missing when acetyl-CoA was omitted from the reaction (– AcCoA, Figure 3f) or when lysine residues were substituted by alanine residues (data not shown).

To map the major site of acetylation in monoacetylated Tat, the ARM peptide was digested with Lys-C, an endoproteinase that hydrolyzes peptide bonds specifically at the carboxylic side of lysine residues. Experiments using synthetic acetylated peptides (acetylated and unacetylated) indicated that acetylation of a lysine residue interfered with its cleavage by Lys-C. Following acetylation of the ARM peptide, a mass increase of 42 Da (addition of one acetyl group) was exclusively detected in the amino-terminal Lys51-specific cleavage fragment, which contained both Lys50 and Lys51 residues (ISYGRKK; Figure 3g). As we did not detect a shift in molecular mass in the carboxy-terminal Lys50-specific cleavage fragment containing the Lys51 residue (KRRQRRRP), we concluded that Lys50 is the predominant site of acetylation.

**Figure 3**

Mapping of the Tat acetylation sites to lysine residues 50 and 51. (a) Tat lysine (K) residues are shown aligned with the Tat protein and its functional domains. Peptides used to map the acetylation site are shown with respect to the Tat open reading frame. (b) Synthetic peptides corresponding to Tat domains, to the amino-terminal tails of histones H3 and H4 and to the T7 and FLAG epitopes were incubated with the catalytic domain of p300 in the presence of [1-$^{14}$C]acetyl-CoA (AcCoA) as described in Figure 2. Reaction products were separated by electrophoresis on Tris-Tricine gels (BioRad) and processed for autoradiography. (c) Peptides corresponding to the amino terminus of histone H4 and to amino acids 45–58 of Tat (Tat 45–58) or to the same region in which Lys50 and Lys51 were replaced with alanines (Tat-AA 45–58) were incubated with the catalytic domain of p300 in the presence of [$^{14}$C]acetyl-CoA and analyzed as described in (b). (d) Wild-type recombinant His$_6$–Tat protein or a mutant in which amino acids 50 and 51 were replaced with alanines (Quickchange site-directed mutagenesis, Stratagene) were incubated in the presence of increasing amounts of GST–p300 with [1-$^{14}$C]acetyl-CoA. Reaction products were separated by SDS–PAGE and analyzed by autoradiography. (e) Synthetic peptides corresponding to Tat ARM (amino acids 45–58; KK), or mutants in which Lys50 (AK), Lys51 (KA) or both Lys50 and Lys51 (AA) were replaced by alanine were incubated with the catalytic domain of p300 in the presence of [1-$^{14}$C]acetyl-CoA. Reaction products were visualized by autoradiography and quantified with a Phosphorimager. (f) Peptide Tat 45–58 was incubated with p300 in the presence or absence of acetyl-CoA. Samples were analyzed by MALDI mass spectrometry on the positive ion mode with delayed extraction on a Reflex II time-of-flight instrument (Bruker-Daltonik GmbH). (g) Peptide Tat 45–58 incubated with p300 in the presence or absence of acetyl-CoA was digested with endoproteinase Lys-C (Roche, peptide:enzyme ratio 10:1, w/v) in 40 mM ammonium bicarbonate at 37°C for 1 h. Digested samples were analyzed by mass spectrometry with the thin-film preparation technique [14].



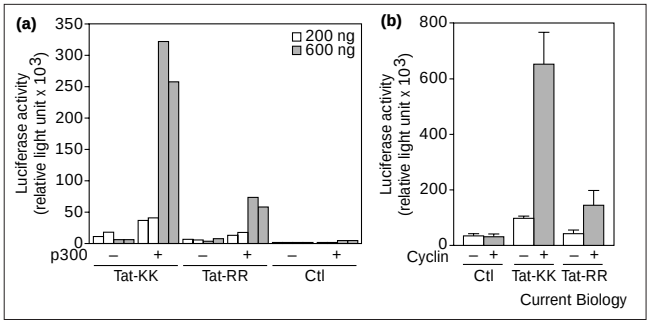

Indeed, analysis by nanospray ion-trap spectrometry of the ISYGRKK fragment isolated after acetylation revealed collision-induced ion products corresponding to the presence of acetylated Lys50 (data not shown). In agreement, the single mutation of Lys50 to an alanine reduced [1-$^{14}$C]acetyl incorporation in response to p300 *in vitro* by more than 60%, whereas the acetylation level was not affected when residue Lys51 was mutated to alanine (Figure 3e). Importantly, mutation of Tat Lys50 and Lys51 to alanine prevented the acetylation of Tat *in vivo* after metabolic labeling of transfected cells with $^{3}$H-labeled sodium acetate (data not shown).

The arginine-rich motif of Tat has been implicated in several functionally important activities. These include binding of Tat to TAR, its target RNA element, nuclear localization, and protein stability. To investigate the functional relevance of Tat acetylation, we mutated both Lys50 and Lys51 to either alanine (Tat-AA) or arginine (Tat-RR) residues in the full-length Tat open reading frame. These mutations did not interfere with protein stability (see Supplementary material). However, indirect immunofluorescence experiments revealed that Tat-AA exhibited partially defective nuclear import (see Supplementary material).

We therefore focused our attention on the Tat-RR mutant, which conserves the positive charge associated with the unacetylated lysine residue. This substitution has been used to assess the role of reversible acetylation in the amino-terminal tails of histone proteins [9]. Substitution of lysine residue 50 by arginine in the ARM of Tat does not affect its affinity for TAR [10] (M.O. and E.V., unpublished observations). Importantly, the same mutations did not modify the subcellular localization of the Tat protein (see Supplementary material). To determine whether acetylation of Tat by p300 is important for the functional synergy observed between both proteins, we cotransfected expression plasmids encoding wild-type Tat (Tat-KK) or Tat-RR with increasing amounts of a plasmid encoding p300 (200 and 600 ng). Substitution of Lys50 and Lys51 with arginines severely reduced the ability of Tat to activate the HIV LTR in synergy with p300 (Figure 4a).

As the molecular mechanism of Tat transactivation of the HIV LTR is based on its interaction with cyclin T1 and

**Figure 4**




Acetylation of Lys50 and Lys51 is critical for the synergy between Tat and its cofactors p300 and cyclinT1. (**a**) HeLa cells were cotransfected with 180 ng NFκB-deficient LTR–luciferase reporter construct, 2 ng full-length $Tat_{101}$-expressing vector (Tat-KK) [8] or 2 ng of a vector expressing a mutated Tat protein in which Lys50 and Lys51 were mutated to arginines (Tat-RR) or no Tat (Ctl) in the presence of 200 or 600 ng of the p300-expressing vector (+ p300) or the empty vector as a control (– p300) [12]. Transfections were performed according to the protocol described in Figure 1. One representative experiment, performed in duplicate, out of four is shown. (**b**) NIH3T3 cells were cotransfected with 100 ng of the wild-type LTR–luciferase reporter construct, 10 ng of the Tat-expressing vector (Tat-KK) or a vector expressing Tat-RR as described above in the presence of a vector expressing the human cyclin T1 protein (+ cyclin) or the empty vector as a control (– cyclin) [1]. Cells were harvested 24 h after transfection and processed in a standard luciferase assay [8]. The average ± SEM of three independent experiments performed in duplicate is shown.

the recruitment of the pTEFb elongation complex, we investigated the role of Lys50 and Lys51 in the Tat–cyclin T1 synergy [1]. Murine cyclin T1 cannot synergize with Tat to enhance Tat affinity for TAR, resulting in deficient Tat transactivation in murine cells [11]. As reported, this defective phenotype was corrected when an expression vector for human cyclin T1 was cotransfected with a Tat expression vector and an LTR reporter construct in mouse NIH3T3 cells (Figure 4b). Mutation of Lys50 and Lys51 to arginines prevented the rescue of Tat activity by human cyclin T1 in murine cells (Figure 4b). This experiment indicates that the conservation of lysine residues that can be acetylated in the ARM region of Tat is important for its ability to synergize with human cyclin T1 to activate the HIV promoter.

These experiments describe a newly identified post-translational modification of the HIV transcriptional activator Tat with important functional consequences. Indeed, mutation of the acetylation-targeted lysine residues of Tat impairs its ability to synergize with p300 or with cyclin T1 to activate the HIV promoter. Future experiments will examine how acetylation of Tat modulates its ability to interact with TAR and Tat cofactors and should contribute to an increase in our understanding of the molecular mechanism of Tat action on the HIV promoter.

*Supplementary material*

Supplementary material including the effects of mutation on Tat stability and subcellular localization is available at http://current-biology.com/supmat/supmatin.htm.

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# Supplementary material

# Acetylation of the HIV-1 Tat protein by p300 is important for its transcriptional activity

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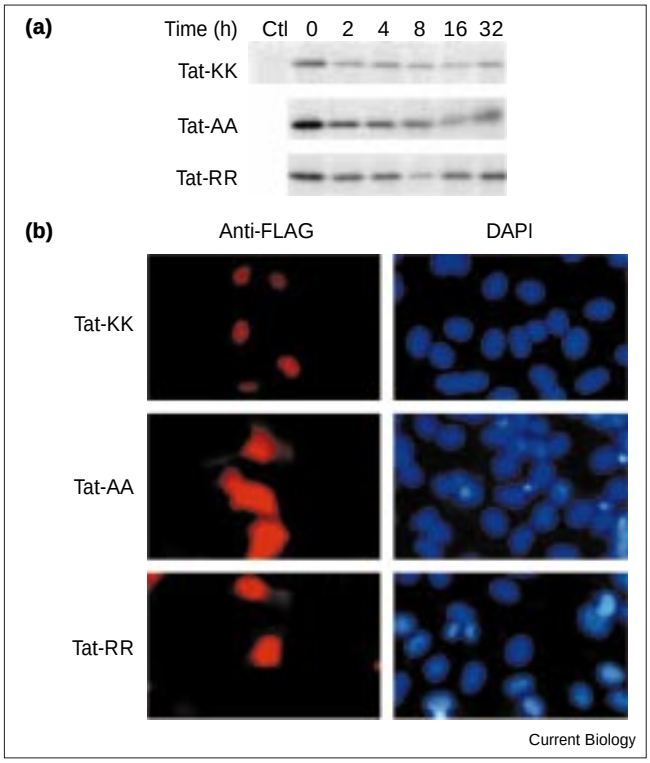
## Supplementary results and discussion

### Characterization of mutant Tat proteins

Mutations were introduced in codons for lysine residues 50 and 51 in expression vectors for epitope-tagged Tat proteins (FLAG at the carboxyl terminus). The wild-type (Tat-KK) and mutant proteins (Tat-AA and Tat-RR) were characterized in terms of stability using pulse-chase analysis (Figure S1a) and in terms of subcellular localization using immunofluorescence microscopy (Figure S1b).

**Figure S1**




Characterization of mutant Tat proteins. (**a**) Pulse–chase analysis. 293 cells were transfected with expression vectors for Tat-KK, Tat-AA and Tat-RR or empty vector (Ctl). Twenty-four hours after transfection, cells were starved for 1 h in methionine/cysteine-free medium followed by overnight incubation in [$^{35}$S]methionine/[$^{35}$S]cysteine (0.2 mCi/ml). Cells were chased for different periods of time (0, 2, 4, 8, 16 or 32 h) in complete medium. Cellular lysates were prepared in 500 µl lysis buffer (250 mM NaCl, 0.1% NP-40, 20 mM $NaH_2PO_4$, 5 mM EDTA pH 8, 30 mM sodium pyrophosphate, 10 mM NaF, 10 mM sodium butyrate), duplicates were pooled, and immunoprecipitations were performed on 1 mg total protein extract with an anti-FLAG antibody (M2, Kodak, 10 µg/ml lysate) and 30 µl 50% protein-G–Sepharose slurry (Pharmacia) for 30 min at 4°C. The immunoprecipitated material was analyzed by SDS–PAGE and autoradiography. Experiments with [$^{35}$S]methionine/cysteine labeling were performed with and without preincubation of the M2-antibody with 1 µg synthetic FLAG peptide and were exposed to X-ray film for 48 h. (**b**) Subcellular localization. Subconfluent HeLa cells, grown overnight on coverslips, were transfected with expression vectors for Tat-KK, Tat-AA or Tat-RR (2 ng DNA/µl) using lipofectamine (Gibco-BRL) according to the protocol described in Figure 1. After 24 h, cells were fixed with 4% paraformaldehyde in phosphate buffer for 15 min at room temperature, permeabilized with ice-cold methanol followed by 0.1% Triton X-100 treatment and blocked with 10% FCS in phosphate buffer. Monoclonal M2-anti-FLAG antibody (Sigma) was used at a final concentration of 3 µg/ml followed by incubation with Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) at a concentration of 1:600, both for 30 min at room temperature. During the last 5 min of the incubation of the secondary antibody, 4′6-diamidino-2-phenylindole (DAPI, Sigma) was included at a final concentration of 10 mg/ml. Stained preparations were examined on a Zeiss Axiophot microscope (100× magnification).