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Real time measurements of elongation by a reverse transcriptase using surface plasmon resonance

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ABSTRACT **A rapid direct assay for polymerase-induced elongation along a given template is an obligate requirement for understanding the processivity of polymerization and the mode of action of drugs and inhibitors on this process. Surface plasmon resonance can be used to follow the association and the dissociation rates of a given reverse transcriptase on DNA·RNA and DNA·DNA hybrids immobilized on a biotin–streptavidin surface. The addition of nucleotides complementary to the template strand produces an increase in the local mass, as deduced from an increase in the measured signal, due to elongation of the primer strand that allows an estimation of both the extent and rate of the polymerization process. The terminator drug 3′-deoxy-3′-azidothymidine triphosphate completely abolishes the increase in signal as would be expected from an inhibition of elongation. This technique provides a sensitive assay for the affinities of different polymerases for specific templates and for the effects of terminators of the elongation process.**

Replication and transcription are key processes in the cell. The enzymes responsible for the processes of copy or retrocopy of the genetic material share common mechanistic features. In particular, their sequence-dependent processivity is likely to be controlled by similar underlying mechanisms. There is thus a clear interest in gaining easy access to those rates governing rate-limiting translocation steps for this class of enzymes.

The mode of action of DNA polymerases and retrotranscriptases able to function using synthetic templates is generally studied by two techniques. Either the accumulation of a defined labeled product is measured as a function of time or the change in the ratios of major populations of products is assessed. Both approaches rely on stopped flow/quenched flow techniques linked to accurate densitometry generally provided by phosphor image technology. Despite improvements such as the introduction of sequence-specific templates hybridized to primers with various structures (1, 2), there are at least two major drawbacks to these approaches. (*i*) Their relative sophistication excludes routine use for scoring purposes. (*ii*) Traffic along a given fragment is assessed uniquely by quantification of product accumulation (cf. ref. 3, for example). A technology is required that allows the recording of binding, elongation, pausing, and dissociation on the same device.

Surface plasmon resonance techniques measure small local changes in concentration at a surface (4, 5).† The technique has been exploited to measure binding affinities between DNA immobilized on a surface and proteins in solution (6). We decided to investigate whether surface plasmon resonance could be used to follow the relatively small changes in mass associated with polymerization and variations in the concentrations of those binary complexes involved.

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We present here a feasibility study that shows that this is indeed possible and that also illustrates how this technology can assess, even on nonoptimal templates, the efficiency of chain terminators during retrotranscription.

MATERIALS AND METHODS

Oligomers. Poly(rA) (average length as deduced from PAGE = 300 bases) was obtained from Boehringer Mannheim. Three of the oligomers plus biotin-5′-$(dT)_{20}$ (Genset, Paris) used in this study were as follows: oligomer 1, 5′-CCAGTGAATCCGTAATCAAAA-3′-biotin; oligomer 2, 5′-GATTACGGATTCACTGGCCGATTCATTAAGCAG; oligomer 3, 5′-$(T)_{25}(G)_{25}$CTGCTTAATGAATCGG.

Proteins. Moloney murine leukemia virus reverse transcriptase (MoMLV-RT) both RNase H plus and minus was purchased from GIBCO/BRL. The concentration in mol of active sites per liter was assessed by measuring the amplitude of a burst of incorporation on a hybrid of defined concentration as explained (7).

Immobilization of Oligomers $(dT)_{20}$·Poly(rA). Surface plasmon resonance measurements were conducted by using a BIAcore instrument from Biosensor Pharmacia. The 5′-biotinylated $(dT)_{20}$ (0.125 μg/ml) in 75 μl was injected across a

Abbreviations: MoMLV-RT, Moloney murine leukemia virus reverse transcriptase; RU, resonance unit(s); AZTTP, 3′-deoxy-3′-azidothymidine triphosphate.

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†Collective resonating oscillations of free electrons at a gold surface (the plasma) produce a transverse wave (surface plasmon) propagating along the plasma surface (amplitude $\mathbf{K}_{sp}$). Light incident through a prism to the gold surface at an angle of total internal reflection θ produces an evanescent wave characterized by a wave vector $\mathbf{K}_{ev}$ whose amplitude varies as the square root of sin θ. At a given θ, the two amplitudes are equal; resonance occurs resulting in a decrease in the intensity of reflected light. Since $\mathbf{K}_{sp}$ is itself a function of the permitivity of the sample, ξ_s, recording the angle at which resonance occurs leads to a very sensitive measure of the permittivity and hence the refractive index (n_1) of the solution ($\xi_s = n_1^2$). The BIAcore machine used in this study measures the angle θ at which resonance occurs. Small changes in θ are directly related to changes in the refractive index in the dextran layer (n_1) compared to that of a quartz prism (n_2) since $\Delta\theta = \sin^{-1}(n_1/n_2)$. Changes in refractive index per mass of material are additive as described by the Clausius Mossotti relationship $(n_1^2 - 1)/(n_1^2 + 2) = N\alpha/3\xi_O$, where α is the polarizability of the molecule and N is the number density, and, therefore, changes in the refractive index may be linked to changes in mass (M) since $N = \rho N_A/M$. Thus small differences in θ are directly related to changes in the local mass at the gold/liquid interface. In practice, this interface consists of a dextran layer upon which molecules are immobilized. The wavelength of light (760 nm) used in the BIAcore apparatus allows efficient use of the evanescent wave up to 300 nm into the solution. The units of measurement are expressed in resonance units (RUs) where a change of 10^{-4}° is equivalent to a change of 1 RU and the machine has an effective dynamic range of 3 or 4 RUs to 30,000 RUs. The actual response in RUs as a function of the change in surface molecule depends to an extent upon the differential refractive index of the solute (see *Results*), but for many globular proteins 1 kRU is equivalent to a change in surface concentration of about 1 ng/mm^2.

streptavidin-pretreated dextran sensor surface *in situ* in the BIAcore apparatus at 5 μl/min in 10 mM Hepes, pH 7.4/150 mM NaCl/10 mM $MgCl_2$ at 37°C. Hybridization of poly(rA) (10 μg/ml) was carried out in the same buffer adjusted to 0.3 M NaCl at 37°C by flowing 40 μl at 2 μl/min across the immobilized $(dT)_{20}$.

Cassette System. The 3′-biotinylated oligomer 1 (1 μg/ml) in 75 μl was injected across a streptavidin-dextran surface at 5 μl/min as described above. Oligomer 2 (10 μg/ml) was injected at 5 μl/min at 37°C across the immobilized oligomer 1. Oligomer 3 (10 μg/ml) was subsequently injected at 5 μl/min at 37°C across the immobilized oligomer 1/oligomer 2 hybrid.

Protein Binding. MoMLV-RT was applied at various concentrations to the different immobilized surfaces in 10 mM Hepes, pH 7.4/150 mM NaCl/10 mM $MgCl_2$ at 37°C. The surface was regenerated by washing either with a 10-μl pulse of 0.5% SDS in 10 mM Hepes, pH 7.4/150 mM NaCl/10 mM $MgCl_2$ for 2 min, which removed all bound protein, or with a 10-μl pulse of 10 mM NaOH, which removed nonhybridized oligonucleotides.

RESULTS

We immobilized a short oligomer via a biotin group at its 5′ end to a streptavidin–dextran surface and then hybridized to this *in situ* a longer complementary oligomer (cf. ref. 8). This hybrid is thus a substrate for reverse transcriptase. If the required nucleotide triphosphates are added with the polymerase to the flow cell, then there should be elongation of the immobilized oligomer along the hybridized template. Our reasoning was as follows: for a globular protein a resonance signal of 1 kRU is expected for the binding of 1 ng/mm^2 (9). If we assume that the signal responds uniquely to a local change in mass (more discussion of this point is given later), then 0.5 ng of a DNA molecule (around 7.5×10^{-14} mol of a 20-mer single-stranded DNA template) immobilized on a 1-mm^2 surface would result in an increase of 500 RU. An extension of one base on all the hybridized primers would then result in an increase of 25 RU. This value is well above the background level (<5 RU) and should be observable. With current surface plasmon resonance technology, data are accumulated at a rate of 5–10 Hz so only in the case of relatively slow polymerases (<10 bases per s) will the incorporation of individual bases be observed. Any event, such as a sequence-dependent constraint or the addition of a terminator drug, would be immediately reported as a deviation from the typical elongation rate for a given template.

Immobilization of dT·rA on a BIAcore Surface. The immobilization of 5′-biotinylated $(dT)_{20}$ to a surface pretreated with streptavidin was carried out as described above. Poly(rA) (average length = 300 nt) was applied to the immobilized dT in the same buffer. Optimum hybridization was achieved after approximately 8 min by using rA at 10 μg/ml at a flow rate of 2 μl/min in 0.3 M NaCl at 37°C. Continuous elution to a constant level showed that the change in total resonance units (R response) had increased by 3108 RU commensurate with the hybridization of approximately 16% of the expected poly(rA) (Fig. 1).

Binding of MoMLV-RT to an Oligo(dT)·rA Hybrid and Elongation with Substrates. Upon addition of MoMLV-RT (82 nM in active sites) to the hybrid surface, binding was observed (Fig. 1).‡ A control experiment performed in the absence of nucleic acid indicated that under the conditions used, a degree of nonspecific binding ($K_d = 5.1 \times 10^{-6}$ M) to the dextran surface alone could be observed (Table 1). Curve fitting for MoMLV-RT binding to the dT·rA surface was carried out according to a biphasic model in which one of the binding modes corresponded to the nonspecific interaction with the dextran. MoMLV-RT (devoid of RNase H activity) interacted specifically with the dT·rA hybrid with a k_a of 8.9×10^4 $M^{-1}\cdot s^{-1}$, a k_d of 9.2×10^{-3} s^{-1}, and thus an overall K_d of 1.0×10^{-7} M (Table 1). The wild-type enzyme appeared to have a somewhat better affinity on this surface with an overall K_d of 4.6×10^{-8} M. This latter value was in good agreement with the K_d value (2.0×10^{-8} M) measured in solution by using a stopped flow (Kintek, State College, PA) apparatus. It should be noted that we did not observe any significant degradation of the rA template with the wild-type enzyme. Furthermore, the presence in the injection mixture of dGTP (1 mM), which was not complementary to the template, did not significantly affect the result.

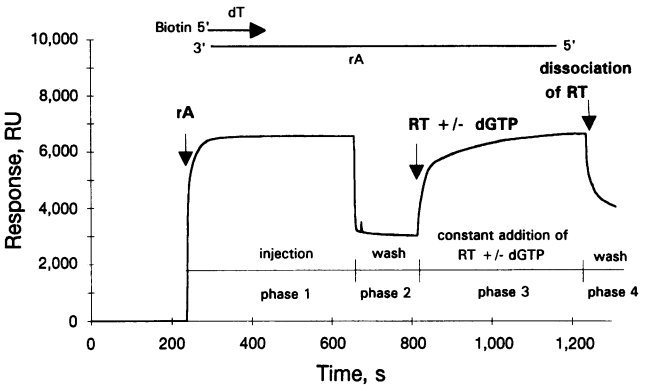

FIG. 1. Hybridization of rA to the immobilized $(dT)_{20}$ and binding of MoMLV-RT(RNase H^-). For $(dT)_{20}$·poly(rA), 5′-biotinylated $(dT)_{20}$ (0.125 μg/ml) in 75 μl was injected across a streptavidin-pretreated dextran sensor surface *in situ* in the BIAcore apparatus at 5 μl/min in 10 mM Hepes, pH 7.4/150 mM NaCl/10 mM $MgCl_2$ at 37°C. Hybridization of poly(rA) (10 μg/ml) was carried out in the same buffer adjusted to 0.3 M NaCl at 37°C by flowing 40 μl at 2 μl/min across the immobilized $(dT)_{20}$ (injection phase 1). After a 2-min wash with 10 ml of buffer (wash phase 2), a solution of MoMLV-RT(RNase H^-) (82 nM) (referred to as RT) and dGTP (1 mM) was injected over the immobilized $(dT)_{20}$·rA hybrid surface (constant injection phase 3). Finally, the surface was washed with buffer (wash phase 4). The abscissa refers to time in seconds and the ordinate refers to changes in the measured angle (θ) at which resonance occurred (RU). The extremely rapid changes observed initially in RU signals were due to changes in the intrinsic refractive indices of the different solutions as they reached the sensor surface. The resonance angle (θ), reflecting the relative $\delta n/\delta C$ at the immobilized surface, was determined outside these initial bulk refractive index changes when the ratio was presumed to be equal independent of the solvent refractive index. Small peaks occurring at the end of the injection phase are due to an internal valve switch leading to a transient pressure change across the surface. At the end of the perturbation the signal returns to the expected level prior to the perturbation.

‡To obtain the rates associated with the formation (k_a) and dissociation (k_d) of a given complex, sensorgrams were fitted to the algorithms provided by the BIAcore instrumentation (10). For the dissociation process (k_d), the rate of change of resonance units (R) as a function of time was fitted to a simple exponential [$R_t = R_o\exp(-k_d t) + R_{drift}$]. The association phase (k_a) was described by the equation: $R_t = [k_a C R_{max}/(k_a C + k_d)]\ \{1 - \exp[-(k_a C + k_d)t]\} + R_{bulk} + R_{drift}t$. The expected response R_t as a function of maximal analyte binding capacity (R_{max}) is calculated as a function of the concentration (C) of added soluble protein. In practical terms, the bulk contribution made by the sample refractive index (R_{bulk}) may be eliminated by careful exchange of the sample into the binding and eluting buffer by using a desalting column on the Smart system (Pharmacia) (11). In cases where this is not feasible, for accurate amplitude measurements (the extent of protein binding or the extent of nucleic acid synthesized), the change in R values is compared at constant refractive index of the solution characteristic of the approach to equilibrium (see figures and also Table 1). Careful temperature control minimized the baseline drift (R_{drift}).

Table 1. Summary of rate constants calculated from sensorgrams

Surface	k_a, $M^{-1}\cdot s^{-1}$	k_d, s^{-1}	K_d, M
MoMLV-RT (RNase H minus) on dextran	$1.7 (\pm 0.3) \times 10^3$	$8.6 (\pm 0.1) \times 10^{-3}$	5.1×10^{-6}
MoMLV-RT (RNase H minus) on $(dT)_{20}\cdot(rA)_{300}$	$8.9 (\pm 0.05) \times 10^4$	$9.2 (\pm 0.1) \times 10^{-3}$	1.0×10^{-7}
MoMLV-RT (RNase H plus) on $(dT)_{20}\cdot(rA)_{300}$	$4.8 (\pm 0.2) \times 10^4$	$2.2 (\pm 0.8) \times 10^{-3}$	4.6×10^{-8}
MoMLV-RT (RNase H minus) on $(dT)_{20}\cdot(rA)_{300}$ during synthesis	$2.9 (\pm 0.02) \times 10^5$	$3.6 (\pm 0.1) \times 10^{-3}$	1.2×10^{-8}
MoMLV-RT (RNase H minus) on multioligomers	$7.6 (\pm 1.0) \times 10^5$	$9.6 (\pm 1.5) \times 10^{-3}$	1.3×10^{-8}

Sensorgrams showing the rate of change of response as a function of time during the interaction of various enzymes with different immobilized templates were produced and analyzed. On rates (k_a) and off rates (k_d) were obtained by reiterative fits of direct linear rate equations describing simple single site binding models.

To monitor a faithful retrocopy of the immobilized primer hybridized to the template, the same experiment was repeated followed by a second injection, where dTTP (1 mM) was substituted for dGTP (1 mM). Under these conditions the preequilibrium of the enzyme (82 nM) with the immobilized hybrid was minimally perturbed (Fig. 2). A steady increase in RU was observed that was attributed to the elongation of the dT primer. The surface was therefore treated with a single 10-μl pulse of NaOH (10 mM) for 2 min that was expected to remove both bound protein and hybridized rA template and then washed with the original buffer. The final baseline level, observed under the same conditions that prevail at the onset of the experiment correspond to a significant increase of 1410 RU arising from the extension of the dT primer. Injection of any nucleotide other than dTTP gave no increase in the signal after this type of serial injection. Furthermore, the extent and the rate of the increase in signal was dependent upon the dTTP concentration. A K_m value of 380 μM was calculated for dTTP and MoMLV-RT on the dT·rA hybrid, a value in agreement with solution measurements for similar templates (3). Association and dissociation rates corresponding to the various phases were computed and are compiled in Table 1. Thus, binding constants can be obtained and elongation can be observed, even on nonoptimal templates immobilized on dextran surfaces.

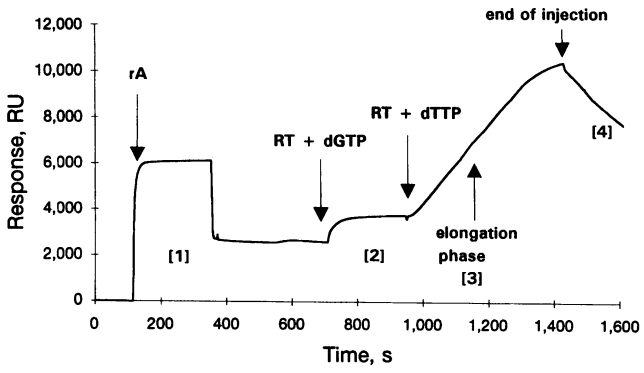

FIG. 2. Injection of MoMLV-RT(RNase H^-) and dTTP at equilibrium. rA was hybridized to the immobilized dT on a sensor surface (phase 1). A solution of MoMLV-RT (82 nM) (referred to as RT) and dGTP (1 mM) was injected across the $(dT)_{20}$·rA immobilized surface (phase 2). After 4 min, a solution of MoMLV-RT and dTTP (1 mM) (phase 3) was coinjected over a 7-min period, followed by buffer alone (phase 4).

Reusable Surfaces and Heterogenous Templates. The poly(rA)/$(dT)_n$ system is inefficient and inconvenient. The double-stranded part of the hybrid is immobilized by the 5′-biotinylated extremity of the primer so that after completion of one efficient run-off experiment the surface itself is saturated. Furthermore, the rA template is heterogenous. Long

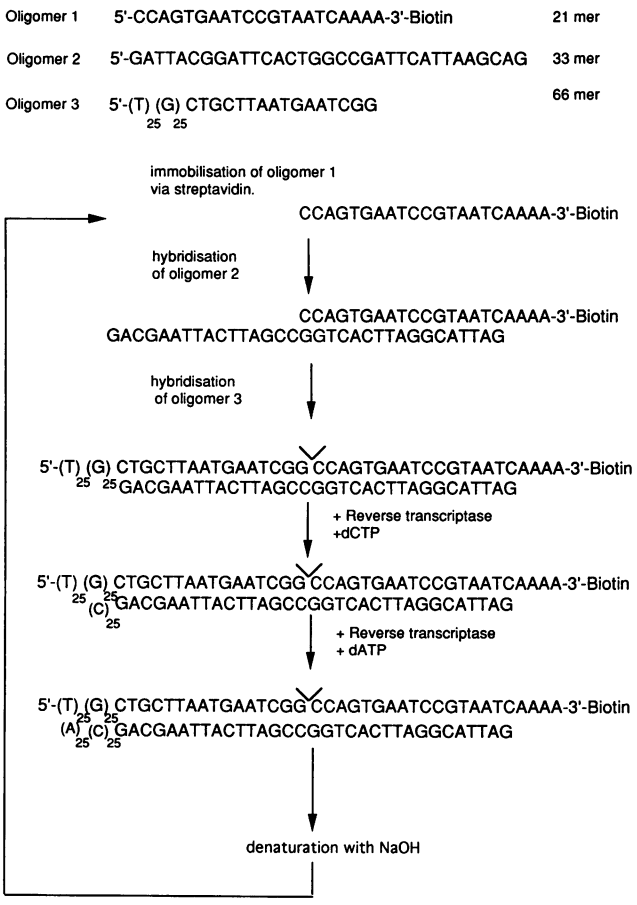

FIG. 3. Scheme illustrating the strategy for the multioligomer system for immobilizing any given template/primer configuration via a reusable oligomer.

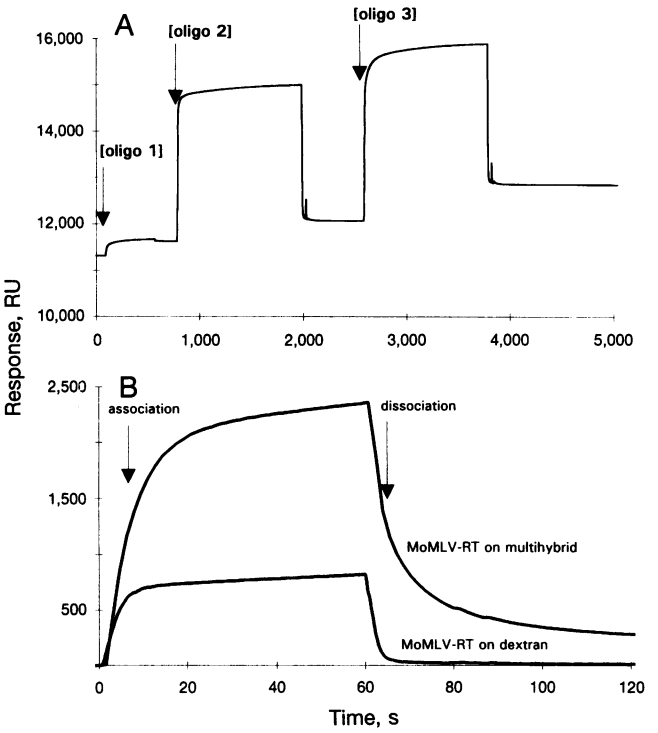

FIG. 4. Immobilization and *in situ* hybridization of multioligomer system and binding of MoMLV-RT. (*A*) Oligomers were flowed across a dextran surface preactivated with streptavidin. Oligomer 1, 3′-biotinylated, in 40 μl of 10 mM Hepes, pH 7.4/150 mM NaCl/10 mM $MgCl_2$ at 37°C was applied at 5 μl/min. Oligomer 2 in 40 μl 10 mM Hepes, pH 7.4/300 mM NaCl/10 mM $MgCl_2$ at 37°C was applied at a flow rate of 2 μl/min. Oligomer 3 in 40 μl 10 mM Hepes, pH 7.4/300 mM NaCl/10 mM $MgCl_2$ at 37°C was applied at a flow rate of 2 μl/min. (*B*) Sensorgrams for MoMLV-RT binding to the multioligomer surface. MoMLV-RT (40 nM) in 10 mM Hepes, pH 7.4/150 mM NaCl/10 mM $MgCl_2$ at 37°C was applied at 5 μl/min to the immobilized multioligomer surface. An identical aliquot was injected across a nonderivatized dextran surface.

nucleic acids clearly experience difficulty in penetrating the dextran layer. Consequently, the majority of reactions occur at the dextran–solvent interface where the evanescent wave is already rather weak, thus diminishing considerably the sensitivity of the system.

To solve some of these problems we devised a renewable multioligomer cassette system that is illustrated in Fig. 3. Oligomer 1 has a 3′ biotin group allowing it to be easily immobilized onto a streptavidin surface. Oligomer 2 has partial 5′ complementarity to oligomer 1 allowing hybridization and leaving a sticky end complementary to the 3′ end of oligomer 3. The complete hybrid then has oligomer 2 as primer to be extended along the template that is the unhybridized 3′ extremity of oligomer 3 (Fig. 3). We designed oligomer 3 so that addition of dCTP in a polymerization context would extend 25 residues and then addition of dATP should extend another 25 residues up to the end of the fragment (Fig. 3).

Immobilization of the 3′-biotinylated oligomer 1 increased the baseline signal by 1350 RU under the conditions described above (Fig. 4*A*). Subsequent addition of oligomer 2 (1624 RU) showed that hybridization went to about 75% completion based upon the expected mass accumulation. Subsequent addition of oligomer 3 (2061 RU) showed that hybridization went to around 70% of that expected.

Binding of MoMLV-RT gave binding curves of the type shown in Fig. 4*B*. The protein–DNA interaction was disrupted by flowing 4 M NaCl (10 μl at 5 μl/min). This reduced the RU response to that of the hybrid cassette. MoMLV-RT bound to the multioligomer with rate constants given in Table 1 (k_a = $7.6 \times 10^5 M^{-1} \cdot s^{-1}$ and $k_d = 9.6 \times 10^{-3}\ s^{-1}$), giving an overall K_d of 1.3×10^{-8} M.

Quantitative Analysis of the Polymerization Process. We used this cassette system first to calibrate our system and to calculate the equivalent ΔRU associated with nucleic acid or protein bound to the dextran. As described above, in a heterogenous system involving large nucleic acids, a complex average for the distribution of the nucleic acid on the dextran surface exists since the dT can hybridize at various positions along the rA template. In the cassette system, the nucleic acid that is synthesized and the protein that is bound are located at the same place and sense the same strength of the evanescent field. We therefore attribute any difference in observed ΔRU per immobilized mass to relative differences in local permitivity at the molecular level. In a typical experiment, a hybrid surface was constructed in which 1463 RU of oligomer 3 had been hybridized. At saturation this surface bound 3716 RU of MoMLV-RT, which at approximately 1 kRU/ng of protein

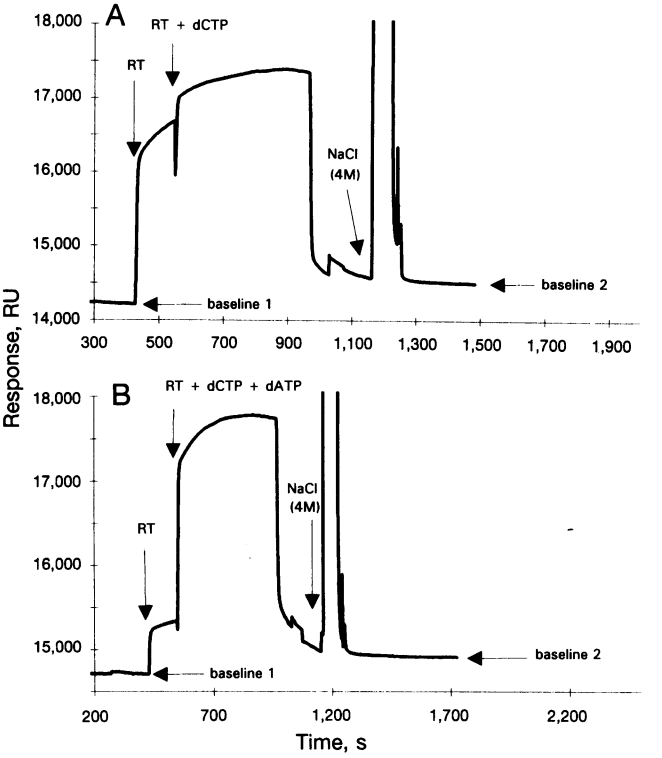

FIG. 5. Elongation of MoMLV-RT along the multioligomer system. (*A*) MoMLV-RT (93 nM) (designated here as RT) was injected at 5 μl/min across the multioligomer surface in 10 mM Hepes, pH 7.4/150 mM NaCl/10 mM $MgCl_2$ at 37°C. A second injection involved MoMLV-RT (93 nM) (designated as RT + dCTP) in the same buffer containing dCTP (1 mM). Note the small peak occurring at the end of the injection phase, due to an internal valve switch leading to a transient pressure change across the surface. A 10-μl pulse of 4 M NaCl for 2 min removed all bound protein but restored the baseline (baseline 2) to 322 RU above the original level (baseline 1). A subsequent injection of MoMLV-RT (93 nM) followed by MoMLV-RT (93 nM)/dATP (1 mM) (designated RT + dATP) produced a NaCl-resistant increase of 308 RU (data not shown). The extended hybrid was removed by a pulse of NaOH (50 mM) at 5 μl/min for 2 min leaving the streptavidin–biotin-bound oligomer 1. (*B*) Oligomers 2 and 3 were rehybridized to the immobilized biotinylated oligomer 1. MoMLV-RT (93 nM) was flowed across the surface followed by a second injection of MoMLV-RT (93 nM)/dCTP (1 mM)/dATP (1 mM) for 7 min at 5 μl/min that gave a NaCl (4 M)-resistant increase of 221 RU. Note again the small peaks occurring at the end of the injection phase, due to an internal valve switch leading to a transient pressure change across the surface. Again, a pulse of NaOH (50 mM) at 5 μl/min for 2 min left the streptavidin–biotin-bound oligomer 1.

(12) is equal to 5.3×10^{-14} mol (3.7 ng) of MoMLV-RT. For stoichiometric binding then, this would correspond to 1.15 ng of oligomer 3, and thus 1 kRU is equivalent to 0.78 ng of oligomer. Strictly speaking, this sets an empirical relationship between the relative change in refractive index (δn) for a change in nucleic acid concentration (δC_{NA}) and that for a protein (δC)$_P$ as $(\delta n/\delta C)_P = 0.78(\delta n/\delta C)_{NA}$. In the Clausius Mossotti relationship described above, the polarizability constant (α) is composed of an induced polarizability and an orientation polarizability; nucleic acids such as DNA have no permanent moment but clearly have a large induced polarizability. A larger $\delta n/\delta C$, as observed, would therefore be expected for a DNA molecule than for a protein.

Elongation along such multioligomer systems was investigated in the experiment described in Fig. 5. Injection of MoMLV-RT (93 nM) followed by MoMLV-RT (93 nM) plus dCTP (1 mM) allowed an increase in synthesis opposite the guanosine sequence (Fig. 5*A*). The surface was then washed with 4 M NaCl. The baseline returned to a level above that of the immobilized oligomer hybrid. Substantial synthesis corresponding to 310 RU was observed. The ΔRU per base could be calculated from the increase in RU measured upon hybridization of oligomer 3 (66-mer). In the case illustrated in Fig. 5*A*, following elongation in the presence of dCTP alone, the ΔRU per base was equal to around 13 RU. This increase corresponded, therefore, to 24 bases and thus 95% of expected synthesis based upon the degree of oligomer 3 hybridization. Repeated addition of MoMLV-RT (93 nM) and dATP (1 mM) gave an RU increase commensurate with the incorporation of 24 bases and thus in accordance with completion of synthesis along the template. Regeneration of the surface with 50 mM NaOH removed oligomers 2 and 3 (oligomer 2 having been extended).

Rehybridization with oligomers 2 and 3 followed by the addition of MoMLV-RT (93 nM) and dCTP (1 mM) and dATP (1 mM) across the hybrid surface in equilibrium with MoMLV-RT gave a NaCl-resistant increase equivalent to an increase of 50 bases (Fig. 5*B*). The injection of MoMLV-RT (93 nM) and dCTP (1 mM) and dATP (1 mM) resulted in a burst in the response due to synthesis along the template. This elongation phase was linear over a 50-s period and the rate of increase was calculated as being 4.4 RU per s. The ΔRU per base associated with the hybridization of oligomer 3 in this experiment was 4.6 RU and thus the average rate of increase could be calculated as being 0.95 bases per s. This correlated also with the duration of the linear phase, which lasted 50 s and again would imply that 50 bases were synthesized as expected along oligomer 3. This rate corresponded exactly with that measured for MoMLV-RT acting under similar conditions on the same template/hybrids in solution. The action of MoMLV-RT is known to be distributive and, although we assume that all correctly docked molecules are going to initiate synthesis simultaneously, it is clear that there will be a distribution of termination/reinitiation events along the nascent primer. However, since we are in excess of enzyme, we believe that the process will be effectively linear until the primer is completely extended at which point the enzyme will be dissociating from the ends. This is in fact observed at the end of the elongation phase where even though enzyme and substrates are continuously flowing across the surface, there is a decrease in the *R* signal.

Efficiency of dT·rA Hybridization on a Dextran Surface. In a typical experiment (cf. Fig. 1) 1253 RU of biotinylated oligomer (dT)$_{20}$ were captured to the surface. Poly(rA) (heterogenous distribution) was applied to the immobilized dT in the same buffer. Optimum hybridization was achieved after approximately 8 min by using rA at 10 μg/ml at 2 μl/min in 0.3 M NaCl at 37°C giving an increase of 3108 RU. On denaturing PAGE, the RNA was established as being around 300 bases long. Thus only approximately 16% of the potential

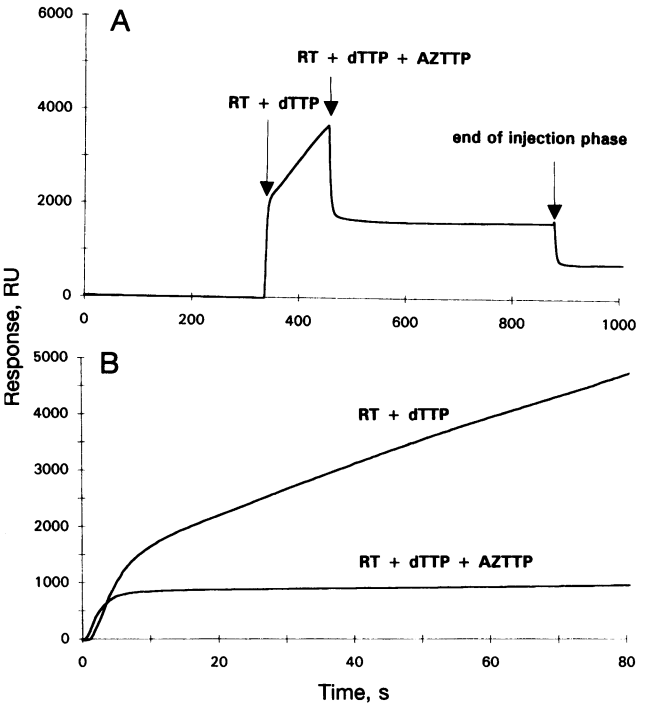

FIG. 6. Effect of the inhibitor AZTTP on elongation. (*A*) MoMLV-RT (82 nM)/dTTP (1 mM) was flowed at 5 μl/min across the (dT)$_{20}$·rA immobilized surface. Two minutes after this initial injection, a second sample containing MoMLV-RT (82 nM), dTTP (1 mM), and AZTTP (10 mM) was injected onto the surface at the same flow rate. (*B*) MoMLV-RT (82 nM)/dTTP (1 mM)/AZTTP (10 mM) was flowed across a fresh dT·rA surface and the difference in the change in RU was compared with that of MoMLV-RT (82 nM)/dTTP (1 mM) alone on another fresh hybrid surface.

dT sites appear to be hybridized. There are two likely reasons for this. (*i*) Accessibility of nucleic acids to the interior of the dextran layer is restricted, more for larger (rA) than smaller [(dT)$_{20}$] molecules. (*ii*) One rA molecule may hybridize to several dT primers. Injection of MoMLV-RT (463 nM) across this surface appeared to saturate the specific binding sites so that 1408 RU of MoMLV-RT were bound, equivalent to 2×10^{-14} mol of protein (according to ref. 9). As demonstrated above and in agreement with ref. 12, the associated change in RU associated with nucleic acid binding to the surface is not the same as that for a protein of equivalent mass, 1 kRU corresponded to 0.78 ng of bound oligomer. The 3108 RU of hybridized rA thus corresponds to 2.5×10^{-14} mol of a 300-base nucleic acid. This simple calculation indicates that more than 80% of the 3′ termini of the immobilized poly(dT) molecules are available for binding and elongation by MoMLV-RT.

Inhibition of Elongation. Injection at equilibrium allows an investigation of the effects of inhibitors of elongation. A solution of MoMLV-RT (82 nM) and dTTP (1 mM) was injected across a freshly hybridized dT·rA surface and again an increase in RU was observed (Fig. 6*A*). After 2 min, a mixture of MoMLV-RT (82 nM), dTTP (1 mM), and 3′-deoxy-3′-azidothymidine triphosphate (AZTTP) (10 mM) was injected onto the surface. An immediate decrease in signal was observed and no further change occurred during the whole of the injection period. The final RU value was an increase of 744 RU with respect to the hybrid alone. After a regeneration pulse with 50 mM NaOH, the original dT had been extended by 790 RU. Again this corresponded to the expected amount based upon the hybridized rA. It appears that synthesis had all but finished after the 2 min prior to the AZTTP injection. It is of note that the AZTTP appears to dramatically increase the

dissociation rate of bound MoMLV-RT. Indeed, injection of MoMLV-RT (82 nM), dTTP (1 mM), and AZTTP (10 mM) directly onto a fresh hybrid surface completely abolished both binding and elongation (Fig. 6*B*).

This technique provides a rapid assay for inhibitors of polymerization along DNA·RNA templates. Equivalent results can be obtained with the more sophisticated heterogenous templates.

DISCUSSION

It is possible to rapidly immobilize small quantities of oligomers to a streptavidin-dextran surface through a biotin molecule at either the 3′ or 5′ extremity of the nucleic acid. Hybridization of complementary strands can then be carried out *in situ* to generate templates for RTs. We can rapidly determine binding constants for a given transcriptase on a specific surface. In the case of the $(dT)_{20}$·poly(rA)-immobilized hybrid, RT bound relatively well, and upon the addition of nucleotide triphosphates, an increase in the *R* signal, associated with a local increase in mass was observed. This increase was dependent on the type of nucleotide triphosphate added and was permanent. At the end of an experiment, the *R* signal did not return to its original level, suggesting that the poly(dT) primer had been considerably extended. If the noncomplementary dGTP nucleotides were added then no increase in *R* signal was observed. We conclude that the RT is capable of carrying out specific elongation of the poly(dT) primer by using the hybridized poly(rA) as template and that this increase is detectable in the BIAcore apparatus. The inclusion of AZTTP completely inhibited this elongation process and, furthermore, increased the dissociation rate of the enzyme. The immobilization, hybridization, binding, and polymerization steps as well as the addition of potential inhibitors are extremely rapid processes clearly demonstrating the potential of this approach in assessing substrates and antitranscription drugs for RTs.

The cassette type system (cf. Fig. 3) allows one to address the question of the specificity of the enzyme for different templates. This requires covalent immobilization via a 3′-biotinylated oligomer. The synthesis of a 3′-biotinylated primer is more involved and expensive than that for a 5′-modified primer. We thus devised a "universal" 3′-modified primer that once immobilized could be used to hybridize any template or series of overlapping templates. Indeed, the inclusion of a third overlapping oligomer that would in fact be the one to be extended permits regeneration of the original surface containing the first oligomer. We could show that MoMLV-RT binds specifically to this immobilized construct and that when challenged with the required oligonucleotides could extend the template.

The system is sensitive to inhibitors of elongation making it ideally suitable for screening potential drugs. A simple regeneration procedure removes the extended oligomer leaving the "universal" 3′ anchor oligomer for subsequent studies without the necessity of changing the sensor surface or removing the sensor chip. This approach lends itself ideally to the study of sequence-dependent polymerization processes such as transcription in which complex elongation and dissociation modes may be studied.

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