A Random-Walk Model for Retardation of Interacting Species during Gel Electrophoresis: Implications for Gel-Shift Assays

Boris P. Belotserkovskii*# and Brian H. Johnston*§

*Cell and Molecular Biology Laboratory, SRI International, Menlo Park, California 94025 USA; #Institute of Molecular Genetics, Russian Academy of Sciences, Moscow 123182, Russia; and §Department of Pediatrics, Stanford University School of Medicine, Stanford, California 94305 USA

ABSTRACT We recently showed that intermolecular DNA triplexes can form during gel electrophoresis when a faster migrating single strand overtakes a slower migrating band containing a duplex of appropriate sequence. We proposed a model to account for the resulting apparent comigration of triplexes with the duplex band when the lifetime of the triplex is much shorter than the time of electrophoresis. The model predicts that short-lived complexes can be detected by a gel-shift assay if the faster migrating component of the complex is labeled, a slower migrating component is in excess, and the complex itself migrates more slowly than either of the components. In this case the labeled component, after dissociation from the complex, overtakes a slower migrating band of the free, unlabeled second component and can be captured by the unlabeled component and again retarded; after dissociation of the newly formed complex the cycle is repeated. If the concentration of unlabeled component in the band is larger than some critical value (c_{cr}), most of the labeled component becomes trapped in this band during the entire time of gel electrophoresis, thus effectively comigrating with the slower migrating unlabeled component. We call this mechanism of comigration "cyclic capture and dissociation" (CCD). Here we present a quantitative analysis of the model of CCD comigration which predicts that CCD comigration can be used not only for the detection of relatively short-lived complexes, but also for estimation of the specificity of complex formation.

INTRODUCTION

The gel-shift assay is one of the most useful methods for investigating macromolecular complexes (for example, see Garner and Revzin, 1981; Fried and Crothers, 1981; Durland et al., 1991; Olivas and Maher, 1994). It is based on the fact that the electrophoretic mobility of a complex is usually lower than that of either of the constituent species, so complex formation can be monitored by separation in the gel. Although there are some important cases in which interaction between complex-forming species in the gel must be taken into account (for a review see Cann, 1996a), in most experiments designed to estimate characteristics of the complex, it is assumed that 1) interaction between the separate components of the complex during gel electrophoresis is effectively excluded because they are rapidly separated due to their different mobilities, and 2) the lifetime of the complex is significantly longer than the time of gel electrophoresis. If these two conditions are met, we refer to the gel-shift assay as "classical." In a classical gel-shift assay, the result of the experiment is completely determined by the conditions of preincubation of the samples before loading on the gel. In this kind of experiment, to determine the dissociation constant, a trace amount of a labeled component (A) is usually mixed with different amounts (but always a significant excess) of the other, unlabeled component (B) and, after incubation, separated by gel electrophoresis. Labeled bands are quantitated, and the relative yield (θ) of the complex is plotted against the concentration of B in the incubation mixture (which is assumed to be practically equivalent to its initial concentration because it is in substantial excess over A). If the time of incubation was long enough to achieve equilibrium, the concentration of B at which $\theta = 0.5$ (midpoint concentration) should be equivalent to the dissociation constant of the complex (Cantor and Schimmel, 1980).

The main limitation of the classical gel shift assay is the requirement that the effective lifetime of the complex (taking into account any possible stabilizing effects of the gel matrix) should be longer than the time of gel electrophoresis, which is usually several hours. Considering the simplicity of performing and interpreting gel shift experiments, it would be useful to develop a gel-shift assay suitable for cases in which the effective lifetime of the complex in the gel is significantly shorter than the time of gel electrophoresis. This can be achieved by the following experiment (Belotserkovskii and Johnston, 1996; Balatskaya et al., 1996).

Let us consider a gel-electrophoretic experiment in which a trace amount of labeled component A is initially localized just at the top of the band (all species are moving from top to bottom) formed by unlabeled component B, which has a lower electrophoretic mobility than A and is in considerable excess over A. Such a situation could arise, for example, if A were loaded onto the gel shortly after B, or if component A were initially in an unstable complex whose intrinsic electrophoretic mobility was less than that of B. Component A (either initially free or having dissociated from the complex) overtakes B and with some probability (which in-

Received for publication 4 April 1997 and in final form 28 May 1997.

Address reprint requests to Dr. Brian H. Johnston at his current address, Somagenics, 460 Page Mill Road, Palo Alto, CA 94306. Tel.: 415-617-1852: Fax: 415-617-1852: E-mail: bhjohnston@aol.com.

0006-3495/97/09/1288/11 $2.00

creases with increasing concentration of B in the band) is captured by B, forming a complex. The complex is retarded relative to B and, after some time, dissociates, forming A, which then overtakes B and again is captured, starting another cycle (Fig. 1). This process (called cyclic capture and dissociation [CCD]) causes retardation of the A-component and may even trap it in the vicinity of the duplex band. At sufficiently large concentrations of B, A and B appear to form a "complex" with the same mobility as B. (We will refer to this as a "pseudocomplex," to distinguish it from the "true" complex AB.) Of course, sooner or later component A will pass through the B-band, resulting in irreversible "decay" of the pseudocomplex during gel electrophoresis, with the characteristic time of "decay" depending on the probability of capturing and, consequently, on the concentration of B in the band.

There are two features of a CCD pattern that can make it more complicated than the pattern of a classical gel shift assay (Belotserkovskii and Johnston, 1996; Balatskaya et al., 1996). First, it may contain, in additional to the pseudocomplex band, a more retarded band corresponding to the true complex, which never dissociates during the time of electrophoresis. Second, a smear, consisting of newly formed complexes, may appear between the pseudocomplex and the true complex. However, these effects are not seen for complexes with lifetimes shorter than a few minutes, which are the objects of interest in the present study. For example, for a preformed complex with a lifetime $\tau_{AB} = 10$ min, the proportion of molecules that never dissociate during the 6 h of gel electrophoresis will be only 2×10^{-16}. Moreover, the characteristic size L of the smear at the upper edge of the pseudocomplex band ($L = \tau_{AB} u_{AB}$, where u_{AB} is the difference between the intrinsic electrophoretic velocities of free B-component and complex AB; see Fig. 1) will be 1/6 mm for a typical value of $u_{AB} = 1$ mm/h. Such a smear would hardly be observable for a typical normal band with a characteristic width of ~1 mm.

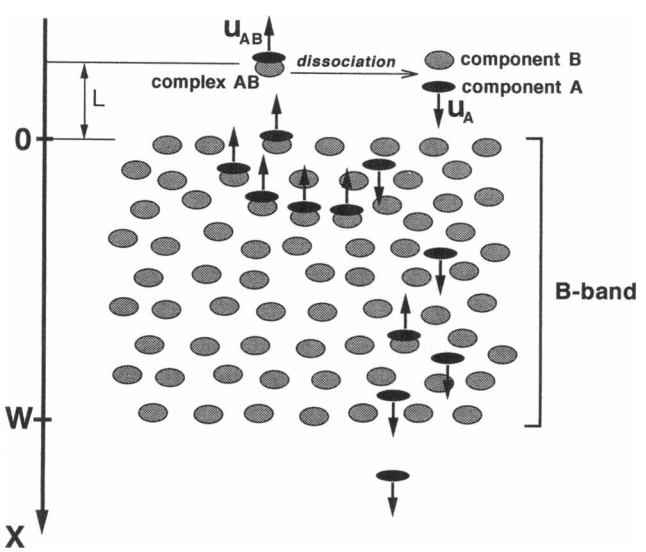

FIGURE 1 Schematic representation of CCD comigration. The movement of the species is considered in the frame of reference of the free B-component (the direction of the gel electrophoresis is from top to bottom). In this coordinate system the complex AB moves upward and the free A molecule moves downward with velocities u_{AB} and u_A, respectively. W is the width of the B-band, and L is the average distance covered by complex AB before its decay.

A simple analysis of the CCD comigration model leads to the conclusion that the faster the on-rate and the slower the off-rate for complex formation, the lower would be the minimum concentration of the B-component required to trap most of the A-molecules within the B-band throughout the time of gel electrophoresis (Belotserkovskii and Johnston, 1996). Thus CCD comigration patterns reflect the specific interaction between A and B. To use CCD comigration for quantitative analysis of complexes, it is necessary to know the dependence of the minimum trapping concentration on the characteristics of the complex and its sensitivity to the time of gel electrophoresis.

For this purpose, we describe here a mathematical analysis of CCD comigration that predicts the main features of this process. Based on this model, we conclude that the interpolated concentration at which half of the A-molecules remain within the B-band at the end of gel electrophoresis (this concentration is an analog of the midpoint in a classical gel-shift assay) should be relatively insensitive to the time of gel electrophoresis in the range of times usually used for gel-shift experiments, and approximates a "critical" concentration that is proportional to the dissociation constant of the complex. Thus in this regard there is a similarity between CCD patterns and classical gel-shift patterns. The model predicts that at concentrations above critical, the time of decay of the "pseudocomplex" increases with decreasing lifetime of the "true" complex AB. Thus the only significant limitation for the detection of complexes by CCD comigration is the technical possibility of creating the critical concentration of B-component in gel.

A system that is formally analogous to that described above was modeled by Eisinger and Blumberg (1973). Their model assumed infinitely rapid equilibrium between species, and as a result, all molecules move inside the B-band with a constant velocity that is calculated as the average weighted velocity over the two states A and AB, which is a function of the concentration of B in the band and the dissociation constant of the complex. Thus this approximation "smoothes out" the irregular, random-walk character of the movement. This approximation holds at concentrations below the critical point (with the best fit for cases in which the mobilities of free B-component and complex AB are close) and allows one to extract the dissociation constant from a measurement of the retardation of the peak of the A-component that passed through the B-band for a given concentration of B-component. However this approximation does not hold near or above the critical concentration, where the pseudocomplex is formed. Our model, presented below, describes the system at all concentrations and leads to the formula of Eisinger and Blumberg (1973) as a limit case.

THE MODEL

In this section we consider two opposing cases of the model. In Part I we completely neglect diffusion in the process of decay of the pseudocomplex; in Part II the role of diffusion is considered. In the first case the problem has a rigorous formulation and solution; in Part II we present arguments that the practical conclusions obtained in Part I remain valid even when the diffusion is the main driving force in the decay of the pseudocomplex.

Part I

Consider the movement of molecule A through the band formed by component B. Because the same molecule A could be either free or within complex AB, we refer to "the molecule in state A" and "the molecule in state AB" instead of "free component A" and "complex AB," respectively.

The distribution of component B in the band is approximated by a zone of constant width W and constant concentration c.

We consider movement in the frame of reference of the B-band (i.e., with velocity v_B). The positive direction is from top to bottom; the upper edge of the B-band is defined as zero; the coordinate is designated x (Fig. 1). In this system a molecule in state A moves toward the bottom with velocity $u_A = v_A - v_B$, and in the state AB it moves toward the top with velocity $u_{AB} = v_B - v_{AB}$. If at one moment the molecule is in state AB, the probability that the molecule will remain in this state after an interval of time Δt is

$$p_{AB}(\Delta t) = \exp(-k_{off}\Delta t) \tag{1}$$

For state A

$$p_A(\Delta t) = \exp(-k_{on}c\Delta t) \tag{2}$$

for $0 < x < W$, and $p_A = 1$ for all other cases.

Note that the molecule always returns to the band some time after crossing the upper edge because of irreversible dissociation of the complex AB in the region above the B-band ("upper space"). In contrast, if a molecule in state A crosses the bottom edge, it will be irreversibly lost from the band.

If $\tau(c)$, which is the average time required for a molecule of A to reach the bottom edge ($x = W$) for a given initial distribution of A-molecules within the B-band, is longer than the time of gel electrophoresis (t_e), most of the A-molecules remain within the B-band at the end of the experiment; otherwise, most of the A-molecules will be lost from the band. Thus the concentration of B-component in the band, which determines which of these outcomes will occur, can be estimated from the equation

$$\tau(c) = t_e \tag{3}$$

To find $\tau(c)$ it is convenient to introduce two new functions, $T_{AB}(x)$ and $T_A(x)$, which are the average times required for a molecule localized initially at point x in state AB or A, respectively, to reach the bottom edge of the band.

Consider the molecule at point x in state AB. Let us choose an interval of time Δt small enough that the probability of the state changing more than once during Δt is negligible compared to the probability of it changing only once. Thus the state will either remain the same during (and at the end of) this interval with probability $p_{AB}(\Delta t)$ (Eq. 1), or change to A during the interval with probability $1 - p_{AB}(\Delta t)$. In the first case at the end of the interval Δt, the molecule is located at the position $x + \Delta x$, where $\Delta x = -u_{AB}\,\Delta t$. In the second case, the molecule moves to the position $x + \Delta x_1$. The exact value of Δx_1 is indefinite because we have not specified the moment at which the state change occurred within the interval Δt, but Δx_1 should be on the same order as Δx. Thus for small intervals of time,

$$\begin{aligned} T_{AB}(x) = \Delta t + p_{AB}(\Delta t)T_{AB}(x + \Delta x) \\ + [1 - p_{AB}(\Delta t)]T_A(x + \Delta x_1) \end{aligned} \tag{4}$$

or, keeping only the terms of first order in Δx,

$$\begin{aligned} T_{AB}(x) = \Delta t + [1 - k_{off}\Delta t][T_{AB}(x) + T'_{AB}(x)\Delta x] \\ + k_{off}\Delta t[T_A(x) + T'_A(x)\Delta x_1] \end{aligned} \tag{5}$$

where T' designates the first derivative.

After the brackets are opened, the term T_{AB} is eliminated. Omitting the terms of order Δt^2 (note that the term that contains Δx_1 disappears) and dividing by Δt, we obtain the linear differential equation

$$u_{AB}T'_{AB} = k_{off}(T_A - T_{AB}) + 1 \tag{6}$$

In a similar way, we obtain for $0 < x < W$,

$$-u_AT'_A = k_{on}c(T_{AB} - T_A) + 1 \tag{7}$$

If a molecule in state A reaches the bottom edge of the B-band, it irreversibly leaves the band. Thus,

$$T_A(W) = 0 \tag{8}$$

If the molecule is located at the upper edge of the band ($x = 0$) in state A, it will move inside the B-band. In contrast, if it is at the same location but in state AB, it will continue moving upward. The average duration of movement upward is equivalent to the lifetime $1/k_{off}$ of the complex, and the average distance covered is

$$L = u_{AB}/k_{off} \tag{9}$$

Then the molecule will change state from AB to A and return to the band in the average time L/u_A. The average total time to return from "upper space" to the B-band will be

$$\tau_r = 1/k_{off}(1 + u_{AB}/u_A) \tag{10}$$

Thus

$$T_{AB}(0) - T_A(0) = 1/k_{off}(1 + u_{AB}/u_A) \tag{11}$$

Using the system of Eqs. 6 and 7 with boundary conditions given by Eqs. 8 and 11, one can obtain $T_A(x)$ and $T_{AB}(x)$ as follows. First we introduce the dimensionless function $f_A = k_{off}T_A$ in place of T_A, dimensionless coordinate $y = xk_{off}/u_{AB}$ (at the bottom edge of the band $y = n$, where $n = Wk_{off}/u_{AB}$), and dimensionless concentration $\chi = (ck_{on}u_{AB})/(u_Ak_{off}) = c\mu/K_d$, where $\mu = u_{AB}/u_A$ and K_d is the dissociation constant of the complex AB. Then

$$f_A(y) = (1 + \mu)[\chi^2/(\chi - 1)^2]\{\exp[(\chi - 1)n] - \exp[(\chi - 1)y]\} + (n - y)(\chi + \mu)/(1 - \chi) \quad (12a)$$

$$f_{AB}(y) = f_A(y) + [(1 + \mu)/(\chi - 1)]\{\chi \exp[(\chi - 1)y] - 1\} \quad (12b)$$

and the average time for traversing the B-band is

$$T_A(y) = f_A(y)/k_{off} \quad (12c)$$

$$T_{AB}(y) = f_{AB}(y)/k_{off} \quad (12d)$$

Using these equations, we can calculate the average time needed to traverse the B-band for any initial distribution of free A-molecules and AB-complexes within the B-band. For simplicity we consider primarily the case in which initially all of the A-molecules are in state A and begin moving down from the top of the B-band. For example, if A were loaded onto the gel after B and overtook B (a so-called chasing experiment; Belotserkovskii and Johnston, 1996), one could obtain

$$\tau(\chi) = f_A(0)/k_{off} = \{(1 + \mu)[\chi^2/(\chi - 1)^2][\exp[(\chi - 1)n] - 1] + n(\chi + \mu)/(1 - \chi)\}/k_{off} \quad (12e)$$

Let us analyze the behavior of function $T_A(y)$ (Eqs. 12a, 12c) in different intervals of χ. If χ is small enough, the term that is linear in y

$$(n - y)(\chi + \mu)/[(1 - \chi)k_{off}] \quad (13)$$

dominates. We refer to this term and the regime in which it dominates as "linear." It is easy to show that this term is simply the time needed to traverse the distance $W - x \equiv (U_{AB}/k_{off})(n - y)$ within the B-band with a constant velocity that is equal to the average velocity (u) of an A-molecule inside the B-band,

$$\underline{u} = (1 - \theta)u_A - \theta u_{AB} \quad (14)$$

where $(1 - \theta)$ and θ are the probabilities of the molecule being in state A or state AB, respectively:

$$\theta = (c/K_d)/[1 + (c/K_d)] \quad (15)$$

To understand how the gel-shift pattern appears in the linear regime for the case in which an A-molecule is initially located at the top of the B-band in state A, let us calculate the distance P by which an A-molecule that has traversed a B-band of concentration χ is retarded in comparison with a control A-molecule that has traversed the same distance in the absence of the B-component ($\chi = 0$):

$$P = u_A[\tau(\chi) - \tau(0)]$$

(Here $\tau(\chi)$ is taken from Eq. 12e.) In the linear regime, this relation can be approximated as

$$P \approx W[(\mu + 1)/\mu][\chi/(1 - \chi)] = W[(1 + \mu)/(1 - \mu c/K_d)]c/K_d \quad (16)$$

(Here n was substituted by Wk_{off}/u_{AB}; see the designations above Eq. 12a.) Thus in the linear regime the retardation is proportional to the width of the B-band and does not depend on the time of gel electrophoresis. Consequently, by choosing a long enough time of electrophoresis, we can separate species (for example, free A and B) by a distance larger than P.

The approximation given by Eq. 16, which becomes more accurate for smaller χ, coincides with the equation obtained by Eisinger and Blumberg (1973) for a formally analogous system in the approximation of infinitely rapid equilibration between species (Eq. A2–A8 in Appendix 2 of the work cited; our μ corresponds to their α, our $1/K_d$ to their K, and our χ to their $\alpha K c_B$). In this approximation (which corresponds to $k_{off} = \infty$), the exponential term in Eq. 12e is 0 at $\chi < 1$, which reduces Eq. 12e to Eq. 13 and leads to Eq. 16 for retardation. (It can be shown that at infinitely rapid equilibration and $\chi > 1$, Eq. 12e gives an infinitely long time for traversing the band. As will be shown in Part 2 of this section, the effect of diffusion must be considered in this case to estimate the time needed to traverse the band.)

It is interesting to note that for $\mu \approx 0$ (i.e., where the mobilities of complex AB and free B-component are very close, which could be the case if the A-molecule is much smaller than B), χ is also approximately zero at any concentration of B, and the formula for retardation reduces to Eq. 16 (which in this case is simply $p = Wc/K_d$), even without the assumption of infinitely rapid equilibration between species.

What happens if the dimensionless concentration χ is larger than 1? Because $\chi = (ck_{on}u_{AB})/(u_Ak_{off})$, at $\chi = 1$ the average distance L covered by complex AB toward the top of the band before its dissociation (u_{AB}/k_{off}) is the same as the average distance covered by a free molecule of A moving toward the bottom of the band before its capture by a molecule of B (u_A/ck_{on}). In other words, at this point the average velocity $\underline{u} = 0$. Note that at $\chi = 1$, concentration $c \equiv c_{cr}$ is proportional to the dissociation constant of the complex

$$c_{cr} = K_d u_A/u_{AB} \quad (17)$$

At concentrations $\chi > 1$ the term

$$(1 + \mu)[\chi^2/(\chi - 1)^2]\{\exp[(\chi - 1)n] - \exp[(\chi - 1)y]\} \quad (18a)$$

dominates.

We refer to this concentration regime and this term as "exponential." [It can be shown that the switch between the linear and the exponential regimes occurs in a narrow interval of width $\sim 1/n$, and the function $f_A(y)$ at the critical point $\chi = 1$ equals $(n^2 - y^2)(1 + \mu)/2 + (n - y)(1 + 2\mu)$.]

The contribution to the functions $T_A(y)$ (Eqs. 12a and 12c) and $T_{AB}(y)$ (Eqs. 12b and 12d) made by the term that depends on the initial position (y) of the molecule A is significantly smaller for the exponential regime than for the linear. For example, for the function $T_A(y)$ in the case of $y = n/2$, for the linear regime this contribution is 50%, whereas for the exponential regime and the same value of y, taking $\chi \approx 2$ and $n > 10$, this contribution is less than 1%. Note that the term that is dominant at large χ,

$$\{(1 + \mu)[\chi^2/(\chi - 1)^2]\exp[(\chi - 1)n]\}/k_{off} \quad (18b)$$

is the same for $T_A(y)$ and $T_{AB}(y)$; thus the time needed to traverse the B-band at large χ depends only weakly on the initial state.

This weak dependence of the time required to traverse the B-band on initial conditions in the exponential regime occurs because the average velocity of the A-molecule inside the B-band is directed toward the top of the band. An A-molecule that happens to be near the bottom edge either escapes from the band or returns to the vicinity of the top edge. If χ is significantly larger than 1, an A-molecule, before it ultimately escapes from the band (which may take a very long time), returns to the top edge of the band many times; thus the "memories" of its initial position and state are lost and the time for escape depends only very weakly on that initial position and state.

The predominantly upward movement of free A-molecules within the B-band should result in A-molecules achieving a quasistationary distribution inside the B-band, with the highest concentration at the top of the band. During electrophoresis these kinetically trapped A-molecules should effectively comigrate with the B-band, imitating a complex with the same electrophoretic mobility as B. We will refer to this metastable electrophoretic fraction, formed by A-molecules that are kinetically trapped inside the B-band, as a "pseudocomplex," to distinguish it from the "true" complex AB.

The term given by Eq. 18b could be interpreted as the lifetime of this pseudocomplex,

$$\tau_{L(pseudo)}(\chi) \approx \{\exp[(\chi - 1)n]\}/k_{off} \quad (18c)$$

or, using $1/k_{off} = \tau_L$ (the lifetime of true complex AB) and $n = Wk_{off}/u_{AB}$,

$$\tau_{L(pseudo)}(\chi) \approx \tau_L \exp[(\chi - 1)W/(\tau_L u_{AB})] \quad (18d)$$

[The preexponential factor $(1 + \mu)[\chi^2/(\chi - 1)^2]$, which is on the order of unity, is omitted.]

The dependence of $\tau(\chi)$, the time needed to traverse the B-band (Eq. 12e), on χ is shown in Fig. 2 for different values of complex lifetime τ_L (*curves 1, 2,* and *3*). The parameter n was calculated as $n = W/(u_{AB}\tau_L)$, and W and

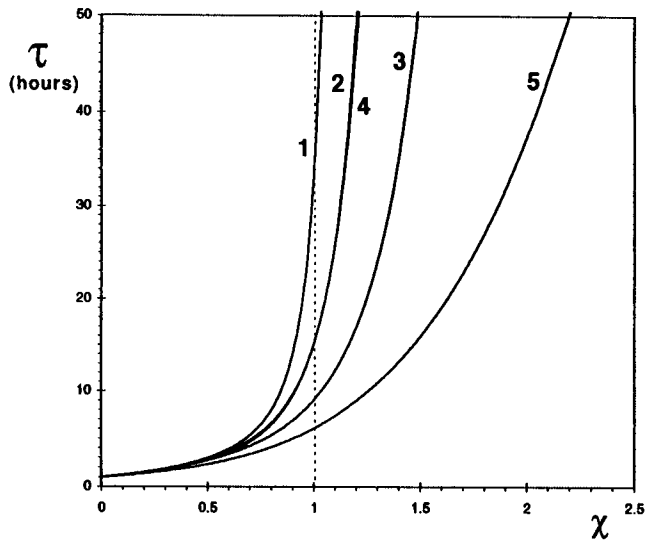

FIGURE 2 The time needed to traverse the B band τ as a function of dimensionless concentration χ. Curves 1, 2, and 3 represent complexes AB with lifetimes τ_L (= $1/k_{off}$) of 2, 5, and 10 min, respectively, with diffusion neglected (see Model, Part I). They were plotted using Eq. 12e for $\mu = 1$. Parameter n was calculated as $W/(u_{AB}\tau_L)$ for $W = 1$ mm and $u_{AB} = 1$ mm/h, yielding $n = 30$, 12, and 6 for curves 1, 2, and 3, respectively. Curves 4 and 5 include consideration of diffusion, but with infinitely rapid interconversion between states A and AB, and were plotted using Eq. 37 with $\mu = \gamma = 1$. The parameter τ_D was 2 min and 5 min, and n_D was 30 and 12, for curves 4 and 5, respectively. These parameters correspond to $W = 1$ mm, $u_{AB} = 1$ mm/h, and diffusion coefficients of 3.6×10^{-2} mm^2/h and 9×10^{-2} mm^2/h for curves 4 and 5, respectively. (These values of diffusion coefficients are in the range obtained by Lunney et al. (1971) for the protein transferrin in polyacrylamide gels.) Curves 2 and 4 nearly coincide in the region shown. The vertical dashed line separates the regions with concentrations above and below the critical concentration $\chi = 1$.

u_{AB} were the same for all curves (see legend to Fig. 2). Because parameter n changes in inverse proportion to the lifetime of the complex τ_L, a decrease in τ_L on the one hand decreases the multiplier before the brackets in Eq. 12e, but on the other hand, it increases the function within the brackets by increasing n. It is easy to show that the second effect overwhelms the first one; thus the shorter the lifetime of complex τ_L, the longer the time needed to traverse the B-band τ for a given χ.

The effect of τ_L on $\tau(\chi)$ is small for concentrations significantly below the critical point (linear regime) and becomes very pronounced near and above the critical concentration (Fig. 2). It is interesting that if in Eq. 12e we interpret n as an "effective number of steps" and τ_L as an "effective time per step," the dependence of the traversing time (τ) on the number of steps (n) is a typical case of the "gambler's ruin" problems (Feller, 1957) (another example is the melting of DNA; Anshelevich et al., 1984). The dependence is linear if the average velocity is directed toward the bottom edge of the B-band (which, in terms of the gambler's ruin problem, is an absorbing barrier); it is exponential if the average velocity is directed toward the top edge of the B-band (a reflecting barrier); and it is quadratic if the average velocity is zero. However, the main distinc-

tion between CCD comigration and the classical random-walk problem is the link, in the case of CCD comigration, between the effective number of steps and the effective time for one step ($n \approx 1/\tau_L$), which in the classical interpretation are independent parameters of the system.

Part II

Before analyzing the role of diffusion in the "decay" of the pseudocomplex, it is necessary to mention that in this case the formulation of the problem is not as rigorous as in the absence of diffusion. First, we ignore the effect of diffusion on the profile of the B-band in the course of gel electrophoresis, because it is impossible to obtain a simple analytical solution for this case. However, we believe that for any change in the profile of the B-band during gel electrophoresis, reasonable upper and lower limits for the traversing time could be obtained by using the approximation that the band has constant width and concentration. Moreover, the effect of diffusion could be diminished (at a given gel porosity and temperature) by increasing the initial width of the B-band, for example, by increasing the volume of the sample loaded.

Another simplifying assumption is that the molecule always returns to the B-band after crossing the upper edge, and never returns after crossing the bottom edge. Furthermore, in the case of short-lived complexes, the distance of penetration of the A-molecule beyond the upper edge should be much smaller than the characteristic size of the B-band. If we neglect this distance, the upper edge of the band could be considered an "impermeable wall" that "reflects" the molecule back to the band (reflecting barrier).

Accepting these simplifying assumptions, we consider the movement of an A-molecule inside a B-band with width W and uniform concentration c of the B-component, and requiring "reflection" at the top edge and "absorption" (loss) at the bottom edge. This formulation of the problem allows us to introduce average times for traversing the B-band, T_A and T_{AB}, as was done in the beginning of Part I of this section.

To generalize Eqs. 6 and 7 for the case in which the diffusion of A-molecules is taken into account, we expand Eq. 4, keeping the terms up to the second order in Δx and then average it over all possible trajectories of A-molecules during the time Δt:

$$T_{AB}(x) = \Delta t + p_{AB}(\Delta t)[T_{AB}(x) + T'_{AB}(x)\langle\Delta x\rangle + T''_{AB}(x) \cdot \langle\Delta x^2\rangle/2] + [1 - p_{AB}(\Delta t)] \cdot [T_A(x) + T'_A(x)\langle\Delta x_1\rangle + T''_A(x)\langle\Delta x_1{}^2\rangle/2] \quad (19)$$

Here the brackets $\langle\ \rangle$ refer to an average over all possible trajectories of the A-molecule during the time Δt. Because for all points inside the B-band the shift due to diffusion is the same in all directions, $\langle\Delta x\rangle = u_{AB}\Delta t$, and $\langle\Delta x^2\rangle = \langle\Delta x\rangle^2 + 2D_{AB}\Delta t$ (Einstein equation), where D_{AB} is a diffusion coefficient of the complex AB. As discussed, $\langle\Delta x_1\rangle$ is on the same order as $\langle\Delta x\rangle$. When all functions in Eq. 19 are expanded in a series on Δt, the terms larger than Δt cancel. Neglecting the terms smaller than Δt and dividing by Δt, we obtain

$$-u_{AB}T'_{AB} + k_{off}(T_A - T_{AB}) + D_{AB}T''_{AB} + 1 = 0 \quad (20)$$

A similar equation can be obtained for T_A:

$$u_AT'_A + k_{on}c(T_{AB} - T_A) + D_AT''_A + 1 = 0 \quad (21)$$

Excluding T_{AB}, we obtain

$$\underline{u}T'_A + [\underline{D} + (u_{AB}u_A/k)]T''_A + [(u_{AB}D_A - u_AD_{AB})/k]T'''_A - [(D_AD_{AB})/k]T''''_A + 1 = 0 \quad (22)$$

Here $\underline{u}$ is the average rate of movement of an A-molecule inside the B-band (see Eq. 14), and D is the average diffusion coefficient:

$$\underline{D} = (1 - \theta)D_A + \theta D_{AB} \quad (23)$$

where $(1 - \theta)$ and θ are the probabilities that the molecule will be in state A or state AB, respectively:

$$\theta = (k_{on}c)/(k_{off} + k_{on}c) = (c/K_d)/[1 + (c/K_d)] \quad (24)$$

and

$$k = k_{off} + k_{on}c = k_{off}[1 + (c/K_d)] \quad (25)$$

What boundary conditions should be chosen in the case of diffusion? As discussed in the beginning of Part II of this section, the bottom edge of the band can be approximated as an absorbing barrier. Thus,

$$T_A(W) = 0 \quad (26)$$

For a molecule initially in state A and located at $x = 0$ (i.e., at the top edge of the band, which we approximate as a reflecting barrier), we obtain

$$T_A(0) = \Delta t + p_A(\Delta t)T_A(0 + \Delta x) + [1 - p_A(\Delta t)]T_{AB}(0 + \Delta x_1) \quad (27)$$

At the reflecting barrier the molecule can move only downward, and at small Δt the dominant component of $\langle\Delta x\rangle$ is $(D\ \Delta t)^{1/2}$. Repeating the same procedures used to obtain Eqs. 20 and 21, we obtain

$$T'_A(0) = 0 \quad (28)$$

We can make Eq. 22 more compact if we introduce the function g, defined as

$$g = T'_A + (1/\underline{u}) \quad (29)$$

Then

$$\underline{u}g + [\underline{D} + (u_{AB}u_A/k)]g' + [(u_{AB}D_A - u_AD_{AB})/k]g'' - [(D_AD_{AB})/k]g''' = 0 \quad (30)$$

If k is large enough (corresponding to short lifetimes and/or large concentration of B-component), the terms in the denominator that contain k will become negligible. [Because k is the reciprocal of the characteristic time of interconversion between states (for example, see Maher et al., 1990), neglecting the terms in the denominator containing k is equivalent to considering an A-molecule as a superposition of states A and AB.] In this case Eqs. 22 and 30 become

$$\underline{u}T'_A + \underline{D}T''_A + 1 = 0 \tag{31}$$

$$\underline{u}g + \underline{D}g' = 0 \tag{32}$$

Applying boundary conditions (Eqs. 26 and 28), we obtain

$$g(x) = (1/\underline{u})\exp(-\underline{u}x/\underline{D}) \tag{33}$$

and

$$T_A(x) = [\underline{D}/(\underline{u})^2][\exp(-uW/\underline{D}) - \exp(-\underline{u}x/\underline{D})] + (W - x)/\underline{u} \tag{34a}$$

$$T_A(0) = [\underline{D}/(\underline{u})^2][\exp(-\underline{u}W/\underline{D}) - 1] + W/\underline{u} \tag{34b}$$

Substituting $g(x)$ as given by Eq. 33 into the different terms of Eq. 30 shows that the ratio of the neglected terms to the retained terms decreases for decreasing values of the dimensionless parameter,

$$\phi^2 = u^2/(kD) = [(u\tau_{ch})/(D\tau_{ch})^{1/2}]^2 \tag{35}$$

Here u and D are the characteristic values of the mobility and the diffusion coefficient, and $\tau_{ch} = 1/k$ is the characteristic time of interconversion between states A and AB. The second representation of ϕ (right-hand side of Eq. 33) shows that it is the ratio between the directed movement made by the A-molecule during the time of interconversion and the undirected diffusion shift during the same time. It can be shown that in the opposite case (i.e., when ϕ is large), Eq. 30 reverts to the diffusionless equation

$$\underline{u}T'_A + (u_{AB}u_A/k)]T''_A + 1 = 0 \tag{36}$$

which is equivalent to the system of Eqs. 6 and 7 obtained in Part I.

Thus the parameter ϕ governs the switching between two pathways for decay of the pseudocomplex. When ϕ is small, the main source of decay is diffusion, and when it is large, the main source is the accumulation of deviations from the average direction of movement, the magnitude of which is inversely related to the rate of interconversion between states. In this case the parameter $(u_{AB}u_A/k)$ plays the role of the diffusion coefficient.

Let us compare the behavior described by Eq. 34b and the previous result (Eq. 12e) where diffusion was neglected. We introduce the ratio of diffusion coefficients $\gamma = D_{AB}/D_A$, dimensionless width $n_D = Wu_{AB}/D_{AB}$, and the time $\tau_D = D_{AB}/(u_{AB})^2$. Then from Eq. 34b we obtain

$$\tau(\chi) = \tau_D\{[(\chi + \mu)(\chi + (\mu/\gamma))/(\chi - 1)^2][\exp[(\chi - 1)n_D/(\chi + (\mu/\gamma))] - 1] + n_D(\chi + \mu)/(1 - \chi)\} \tag{37}$$

In this case τ_D plays the same role (i.e., the time for a single step of walking) as the lifetime $\tau_L = 1/k_{off}$ of the AB complex in the diffusionless model (Eq. 12e). Note that the shorter the lifetime of the complex, the better the approximation given by Eq. 37 fits the process of CCD comigration. Thus τ_D can be considered as the time needed for one step of walking for an infinitely small lifetime of the complex AB. The effective number of steps in this case is given by $n_D = Wu_{AB}/D_{AB}$ (instead of $n = Wk_{off}/u_{AB}$, as in the diffusionless approximation). Note that in Eq. 37, in contrast with the diffusionless model (Eq. 12e), the time needed to traverse the B-band achieves saturation with increasing concentration of B-component in the band:

$$\tau(\chi = \infty) = \tau_D(\exp n_D - 1) \sim \tau_D \exp n_D \tag{38}$$

This limit exists because even if the concentration of B in the B-band were large enough to keep an A-molecule in the AB-state practically all the time, the A-molecule would still be able to cross the bottom edge of the band because of diffusional movement of the complex AB.

Thus Eq. 38 represents the absolute upper limit for the "lifetime" of the pseudocomplex, which is independent of the rate constants of "true" complex formation and dissociation. For example, for curve 5 in Fig. 2, this limit is several thousands of hours. (Of course, this does not mean that a metastable distribution of A-molecules within the B-band could exist for such a long time, because for such a long period the assumption of constant width of the B-band would not hold. Such a long lifetime should be interpreted as being reciprocal to the rate constant for decay of the pseudocomplex.)

Note that the solution of the system will not change significantly if the reflecting barrier, instead of being at the top edge of the B-band (x = 0), were somewhere above it ($x < 0$). If we "allow" the molecules to pass the top edge of the band, the differential equation for T_{AB} will be the same as for inside the B-band (Eq. 20); T_A will be

$$u_AT'_A + D_AT''_A + 1 = 0$$

instead of Eq. 21, and functions T and T' must be continuous at $x = 0$. It can be shown that for reflection at $x = -\infty$ instead of $x = 0$, the preexponential factor in Eqs. 34a, 34b, and 37 will be multiplied by $1 - (u/u_A) = \chi(1 + \mu)/(\chi + \mu)$, which does not significantly change the properties of the function $\tau(\chi)$.

The most important feature seen, regardless of whether diffusion is considered, is the critical point at $\chi = 1$. The functions given by Eqs. 12e and 37 at concentrations significantly below the critical point (linear regime) nearly coincide (see Fig. 2), because the average rate of movement

does not depend on diffusion. Beyond the critical point $\tau(\chi)$ given by Eq. 37, the rate grows less steeply than in the diffusionless approximation for the same effective number of steps and effective time per step (for example, compare *curves 1* and *4*, or *2* and *5*, of Fig. 2). However, for realistic choices of parameters, the lifetime of the pseudocomplex becomes much longer than the usual time of gel electrophoresis for concentrations of B that exceed the critical point by a sufficiently small amount. This steep increase in $\tau(\chi)$ at supercritical concentrations makes the minimum concentration of B for which most A-molecules remain within the B-band throughout electrophoresis (Eq. 3) relatively insensitive to the time of electrophoresis (for a gel-shift experiment with typical B-concentration steps from one lane to the next).

Fig. 3 shows estimations of the average position of the A-molecule within the electrophoretic pattern predicted by this model. It is seen that in the usual gel-shift experiment, when the concentration of the B-component changes by at least a factor of 2 for successive lanes, the switching of the final position of the A-molecule from close to free A to close to free B occurs within one increment in the concentration of B (from $\chi = 2/3$ to $\chi = 4/3$); hence the critical concentration could be estimated as the average of these two concentrations.

It is interesting to note that the system of Eqs. 20, 21, 26, and 28 also can be used to calculate the time of diffusion of a DNA fragment from a matrix of oligonucleotides immobilized within a polyacrylamide gel (such a system is used for DNA sequencing by hybridization; Khrapko et al., 1991; Livshits et al., 1994). In this case, the free DNA fragment plays the role of A, the immobilized oligonucleotide and its complex with the fragment play the roles of B and AB, respectively (thus $u_{AB} = 0$ and $D_{AB} = 0$), the thickness of the gel matrix plays the role of the width W of the band, and the depth in the gel corresponds to $W - x$. If no electric field is applied to the matrix, u_A is also 0. In this case the solution of the system is

$$T_A(x) = [1 + (c/K_d)](W^2 - x^2)/D_A$$

$$T_{AB}(x) = [1 + (c/K_d)](W^2 - x^2)/D_A + (1/k_{off})$$

If initially all fragments are bound to the oligonucleotide and are uniformly distributed inside the matrix, then the average time for escape is

$$\tau(c) = \left[\int_0^W T_{AB}(x)dx\right]/W$$

$$= [1 + (c/K_d)](W^2/D_A)(2/3) + (1/k_{off})$$

This result coincides with the formula for the characteristic time of retarded diffusion (equation 6 of Livshits et al., 1994; in the designation used by Livshits et al., m corresponds to c, K corresponds to $1/K_d$, and τ' corresponds to $1/k_{off}$), except that the numerical coefficient before the first term is 2/3 instead of $(2/\pi)^2$, which is not important, because it is derived from a fitted parameter.

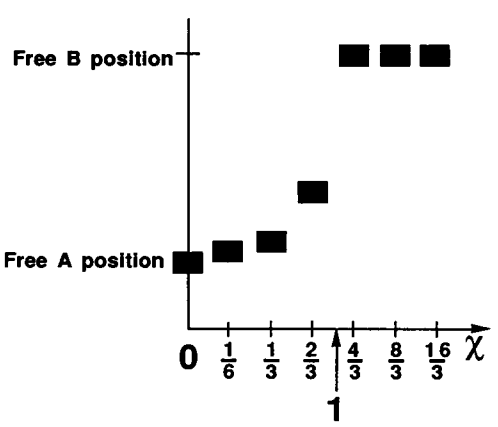

FIGURE 3 Expected retardation of an A-molecule, initially located at the top of the B-band, by the end of gel electrophoresis as a function of concentration of B-component in the band. This diagram shows the expected gel-shift pattern for a series of samples with increasing concentrations of B-component. The retardation was calculated as $p = u_A[\tau(\chi) - \tau(0)]$ if $\tau(\chi)$ is smaller than the time of gel electrophoresis t_e; otherwise, $p = u_A t_e$. The values $\tau(\chi)$ were taken from curve 2 of Fig. 2, and 10 h was used as the time of electrophoresis.

DISCUSSION

In this paper we have analyzed the retardation resulting from interaction in the gel (CCD comigration), for which a general scheme is shown in Fig. 1. We concluded that above some critical concentration of the B component $c_{cr} = K_d u_A/u_{AB}$ (Eq. 17), the A-molecules circulate inside the B-band, migrating as if they were a distinct complex (the pseudocomplex) with the same electrophoretic mobility as B.

The critical concentration has a simple physical interpretation: at subcritical concentrations the average velocity of the A-molecules is directed toward the bottom edge of the B-band, beyond which the molecules irreversibly escape from the band, whereas at supercritical concentrations the average velocity is directed toward the top edge of the band, and upon reaching it the molecules return to the band. At supercritical concentrations, an A-molecule has two pathways for irreversible escape from the B-band (i.e., decaying of the pseudocomplex). The first pathway begins by accumulating deviation from the average velocity. The smaller the characteristic time of interconversion between states A and AB ($\tau_{ch} = 1/(k_{off} + k_{on}c) = \tau_L/[1 + (c/K_d)]$, where τ_L is the lifetime of the "true" complex AB), the smaller the inclination from the average velocity; thus the time for escape through this pathway approaches infinity with decreasing lifetime of the complex AB and increasing concentration of B component in the band. (Here the kinetic constants refer to the conditions of gel electrophoresis; see below.)

The second pathway for escape from the band is diffusion. This pathway gives finite times for escape, even in the

case of infinitely fast interconversion between states. In the previous section we obtained times for traversing the band when each of these pathways predominates, and determined the parameter ϕ (Eq. 35), which characterizes the point of cross-over between these cases. Although we have not obtained a solution for the more complicated intermediate case ($\phi \approx 1$), it is reasonable to assume that these pathways are working essentially independently; thus the time for escape from the B-band could be estimated as the shorter of the times given by Eqs. 12e and 37.

In both cases, at concentrations that only slightly exceed the critical concentration, the length of time an A-molecule is trapped inside the B-band becomes longer than any practical time of gel electrophoresis (Fig. 2). Thus the only practical limitation for complex detection by CCD comigration is the ability to achieve concentrations of B in the gel of approximately c_{cr} without overloading of the gel.

What is the range of lifetimes of complexes (τ_L) that could be detected and analyzed by CCD comigration? Typical values of on-rate constants for triplex formation in the presence of magnesium ions are $\sim 10^3$ M^{-1} s^{-1} (Maher et al., 1990; Faucon et al., 1996; Balatskaya et al., 1996), although they can exceed 2×10^4 M^{-1} s^{-1} (Rougee et al, 1992). In our experiments with triplexes (Belotserkovskii and Johnston, 1996; Balatskaya et al., 1996), we did not observe any nonspecific retardation at concentrations of the target duplex (the B-component) in the sample up to at least 10^{-5} M. Thus, if the concentrations and on rates in the gel are on the same order as in the sample, triplexes with lifetimes of ~ 1 min or even less could be detected by CCD comigration. For complexes based on Watson-Crick recognition ($k_{on} \approx 10^6$ M^{-1} s^{-1}; Wetmur, 1991), the minimum lifetime for detection predicted by our model would be less than 1 s.

In general, concentration of the B-component in the gel band could be either larger or smaller than in the sample, because on one hand, the sample is concentrated upon entering the gel, but, on the other hand, it might be diluted in the well before entering.

One of the most important aims of gel-shift assays, however, is to determine the specificity of complex formation, i.e., the free energy difference between a complex with a matched target and one with a target containing one or more mismatches. This free energy difference is calculated from the ratio of the dissociation constants of the complexes:

$$\Delta\Delta G = RT \ln(K_{d\ mismatch}/K_{d\ match}) \qquad (39a)$$

The values of the rate constants and, consequently, the dissociation constant for a given complex might be different between the gel and free solution, even under the same buffer conditions. (This phenomenon is discussed by Fried and Crothers (1981), Revzin et al. (1986), Cann (1989), and Fried and Liu (1994).) However, because the general physical properties (such as size, charge, or shape) are likely to be very similar for these targets and complexes formed with them, the correction in rate constants for gel conditions relative to solution conditions will be very similar for complex formation with these targets. Consequently, the ratio of dissociation constants of these complexes in the gel is expected to be close to its value in solution. Furthermore, if samples of the same volume are loaded onto the same gel, the ratio of concentration of component B in the gel to that in solution is expected to be the same for all samples. Taking into account the likelihood that (u_A/u_{AB}) ratios will be close for the two complexes, the difference in free energies could be estimated as

$$\Delta\Delta G = RT \ln(c_{cr,\ solution,\ mismatch}/c_{cr,\ solution,\ match}) \qquad (39b)$$

The concentrations in this formula are concentrations in the sample (before loading on the gel) that produce critical concentrations in the gel.

Note that in this study we have not considered changes in the concentration of B-component in the B-band during gel electrophoresis because of diffusion. The effect of diffusional dilution of the B-component should manifest itself in the case in which the concentration of B in the band decreases from above to below the critical concentration during electrophoresis. In this case the A-molecules at the end of gel electrophoresis will be located somewhere between the B-band and the free A position, perhaps distributed as a smear. In practice, such a distribution sometimes is observed in the CCD gel-shift patterns at the transition concentration of the B-component (see Fig. 4). However, if such a confusing pattern appears only in one gel lane within a concentration series (for example, see Fig. 4 *B*, *bottom panel*), it does not introduce significant additional error in the estimation of the critical concentration.

In general, the effect of diffusion on the distribution of the B-component in the gel could be decreased by increasing the initial width of the band (by increasing the sample loading volume), decreasing the gel porosity, or decreasing the temperature. Moreover, at least in the case of some proteins, the diffusion coefficient decreases rapidly with increasing ionic strength of the electrophoresis buffer (Lunney et al., 1971).

We think that the relation between the ratio of the critical concentrations and the difference in the free energies (Eq. 39b) is also applicable in cases in which dissociation of the complex in the gel is described by the "cage effect" theory (Fried and Crothers, 1981; Cann, 1989; Fried and Liu, 1994). This theory predicts that each molecule of the complex is confined to a small "cage" or "tube" formed by the gel matrix, and the complex may dissociate and reassociate many times before one of the components escapes from the cage; thus the effective off-rate constant in this case is proportional to the dissociation constant of the complex in solution and to the rate of diffusion from the cage. The effective lifetime of the complex in the cage should always be longer than the lifetime of the same complex in the solution, but may be shorter than the time of the gel electrophoresis, in which case CCD comigration could take place. Note that in terms of our CCD comigration model,

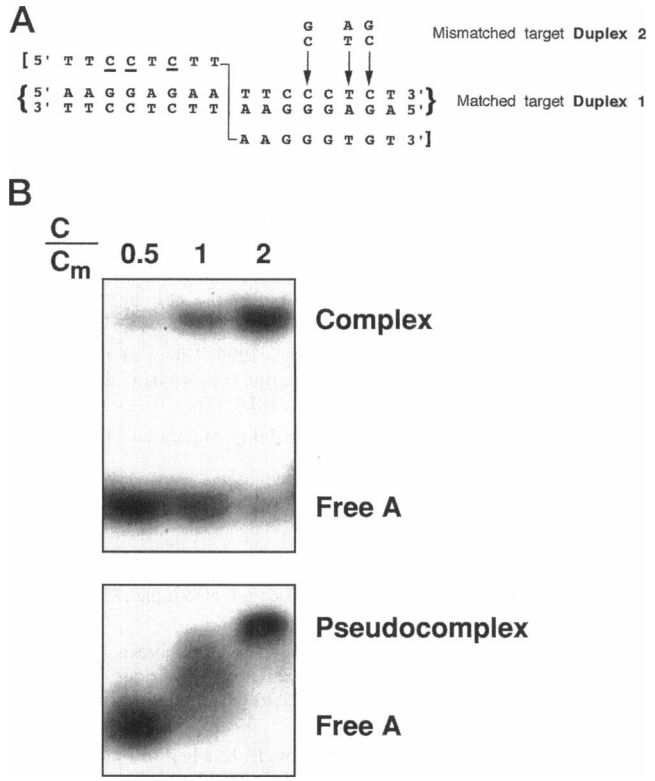

FIGURE 4 Experimental gel-shift patterns for true complex formation and for the pseudocomplex. (*A*) The structure of the complex. The B-component in this case is a Watson-Crick duplex (*between curved braces*), and the A-component is an oligodeoxyribonucleotide (*between square brackets*; C symbolizes 5-methylcytosine), which forms a triplex (i.e., complex AB) with the duplex. We designated this oligonucleotide as AT-16 (Balatskaya et al., 1996). Two types of duplexes were used in the experiment: duplex 1 (matched target), which can potentially form Hoogsteen (or reverse Hoogsteen) pairs with all bases of AT-16, and duplex 2 (mismatched target), which differs from duplex 1 by three inversions (shown by *arrows*), which result in a less stable triplex with AT-16 than that formed using duplex 1. A detailed description of this system is given by Balatskaya et al. (1996). (*B*) Gel-shift pattern for AT-16 as the A-component with duplex 1 (*top*) or duplex 2 (*bottom*) as the B-component. Radioactively labeled AT-16 (5×10^{-10} M) was incubated with different concentrations c (always in significant excess over AT-16) of either duplex 1 or 2 under conditions favorable for triplex formation [10 mM $Mg(OAc)_2$, 40 mM NaOAc (pH 5.1) at 4–6°C]; then samples were electrophoresed through a 10% polyacrylamide gel with the same running buffer. The duplex concentrations are normalized relative to the concentration c_m at the midpoint of the reaction, which is 1.3×10^{-8}M for duplex 1 and 333×10^{-8}M for duplex 2. This 300-fold difference in c_m is due to the lower stability of the triplex formed with mismatched duplex 2 than that formed with duplex 1 (Balatskaya et al., 1996). For duplex 1, two well-defined fractions, free AT-16 (A-component, declining with increasing Duplex 1) and the triplex (complex, increasing with increasing duplex 1), are clearly seen at all concentrations, with no intermediate fraction or smearing at the transition point between free AT-16 and the triplex. A control lane with labeled duplex showed that this complex is strongly retarded compared to free duplex (data not shown). These features of the pattern are typical for a classical gel-shift assay (see Introduction). In contrast, in the bottom panel only free AT-16 is seen below the midpoint, and only the retarded fraction is seen above it. This fraction was shown to comigrate with the duplex (Belotserkovskii and Johnston, 1996). At the midpoint there is a smear instead of two equal and distinct fractions, as expected for the classical gel-shift assay. We previously presented (Belotserkovskii and Johnston, 1996) the evidence that the pattern shown in the bottom panel is typical for CCD comigration. Thus the retarded fraction is a pseudocomplex. This difference in behavior of triplexes formed by AT-16 with duplex 1 and duplex 2 is due to the shorter lifetime of the second triplex because of the presence of mismatches. Note that a large midpoint concentration of the B-component (~0.3 μM for AT-16 with duplex 2) is not an absolute requirement for pseudocomplex formation. For example, for the oligonucleotide dTTCCTCTT, which forms a pseudocomplex with both duplexes 1 and 2, the midpoint concentration for both duplexes is ~60-fold smaller than for AT-16 and duplex 2 (Balatskaya et al., 1996).

the decay of the complex through the cage effect mechanism should still be described as decay of the "true complex," because reassociation inside the cage occurs only between molecules that were initially in the same complex. The penetration by one molecule of a cage containing another molecule followed by their association was not analyzed in detail in the theory of the cage effect, because in the type of experiments for which this theory was developed the rate of reassociation is negligible because of a low concentration of reactants in the gel (Cann, 1996b). However, it is reasonable to assume that this rate (which should play the role of the on rate of complex formation in terms of our model) depends on the properties of the molecule in the same way as the rate of diffusion from the cage. Consequently, the ratio of effective on- and off-rate constants in the case of the cage effect should be proportional to the dissociation constant of the complex, and Eq. 39b is still applicable.

Note that it is very easy to distinguish a pseudocomplex produced by CCD comigration from a complex that does not decay at all during the time of electrophoresis because of the cage effect. In the case of the cage effect, the average mobility of a molecule of A involved in complex formation should be close to the intrinsic mobility of the complex AB, because most of the time the molecule remains within the complex. In contrast, the mobility of the pseudocomplex is the same as the intrinsic mobility of the B-component and is independent of the intrinsic mobility of the complex. (It is assumed that the B-component is in significant excess over A; if the amounts of A- and B-components are comparable, the CCD pattern is expected to be more complicated, because in this case the distribution of B within the band will be noticeably skewed toward lower mobility because of participation in complex formation.)

To what types of systems might CCD comigration be applied? We have observed CCD comigration in the case of intermolecular triplexes (Belotserkovskii and Johnston, 1996; Balatskaya et al., 1996; see the legend to Fig. 4 for a brief description of those findings). In this system the target duplex (the mobility of which is larger than that of the triplex and smaller than that of the free third strand) was the B-component, and the radioactively labeled third strand was the A-component. The formation of pseudocomplex (which we called the T1 form of triplex) was shown to be target-specific. The concentration of duplex in the loaded samples at which a transition to the pseudocomplex was seen, varied from 1×10^{-7} to 67×10^{-7} M for different triplexes, and

in one case was barely observable at 333×10^{-7} M, suggesting that at least up to this duplex concentration, interactions in the gel are specific. (Ambient conditions, volumes of samples, and gel parameters were the same in all experiments.)

We believe that CCD comigration can also be observed in the case of Watson-Crick interactions between relatively short single-stranded regions of different molecules, for example, RNA-RNA interactions. Theoretically, CCD comigration is possible for any macromolecular complexes, if the requirement $v_{AB} < v_B < v_A$ is satisfied.

In conclusion, we note that CCD comigration represents a particular class of systems in which chemical reactions are coupled with gel electrophoresis (for a review, see Cann, 1996a). In the general case, the prediction of the behavior of such systems requires a numerical solution of partial differential equations. However, CCD comigration, with some simplifying assumptions, can be treated as a "gambler's ruin" problem (Feller, 1957) in which the random walk of an object between reflecting and absorbing barriers is considered. This treatment allows us to obtain the dependence of the time needed to traverse the B-band by the A-molecule (see Fig. 1), which is the main parameter determining the electrophoretic pattern, on both the inherent properties of complex AB (on-rate and off-rate constants for complex formation) and the properties of the particular experimental system (velocities and diffusion coefficients of the components in the gel, and the width of the electrophoretic band). The main prediction of the model is that the ratio of dissociation constants of similar complexes and, consequently, the specificity of the complex formation can be extracted from the CCD comigration patterns.

Supported by National Institutes of Health grant R01GM48863 to BHJ.

REFERENCES

Anshelevich, V. V., A. V. Vologodskii, A. V. Lukashin, and M. D. Frank-Kamenetskii. 1984. Slow relaxational processes in the melting of linear biopolymers: a theory and its application to nucleic acids. *Biopolymers.* 23:39–58.

Balatskaya, S. V., B. P. Belotserkovskii, and B. H. Johnston. 1996. Alternate-strand triplex formation: modulation of binding to matched and mismatched duplexes by sequence choice in the Pu-Pu-Py Block. *Biochemistry.* 35:13328–13337.

Belotserkovskii, B. P., and B. H. Johnston. 1996. Capture in the gel: intermolecular triplex formation during gel electrophoresis. *Electrophoresis.* 17:1528–1534.

Cann, J. R. 1989. Phenomenological theory of gel electrophoresis of protein-nucleic acid complexes. *J. Biol. Chem.* 264:17032–17040.

Cann, J. R. 1996a. Theory and practice of gel electrophoresis of interacting macromolecules. *Anal. Biochem.* 237:1–16.

Cann, J. R. 1996b. Comment on mobility-shift computations featuring cage effects. *Electrophoresis.* 17:1535–1536.

Cantor, C. R., and P. R. Schimmel. 1980. Biophysical Chemistry, Vol. 3. W. H. Freeman, San Francisco. 864.

Durland, R. H., D. J. Kessler, S. Gunnell, M. Duvic, B. M. Pettitt, and M. E. Hogan. 1991. Binding of triple helix forming oligonucleotides to sites in gene promoters. *Biochemistry.* 30:9246–9255.

Eisinger, J., and W. E. Blumberg. 1973. Binding constant from zone transport of interacting molecules. *Biochemistry.* 12:3648–3662.

Faucon, B., J.-L. Mergny, and C. Helene. 1996. Effect of third strand composition on triple helix formation: purine versus pyrimidine oligodeoxynucleotides. *Nucleic Acids Res.* 24:3181–3188.

Feller, W. 1957. An Introduction to Probability Theory and Its Applications. Wiley, New York.

Fried, M., and D. M. Crothers. 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* 9:6505–6525.

Fried, M. G., and G. Liu. 1994. Molecular sequestration stabilizes CAP-DNA complexes during polyacrylamide gel electrophoresis. *Nucleic Acids Res.* 22:5054–5059.

Garner, M. M., and A. Revzin. 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system. *Nucleic Acids Res.* 9:3047–3060.

Khrapko, K. R., A. A. Khorlin, I. B. Ivanov, B. K. Chernov, Yu. P. Lysov, S. K. Vasilenko, V. L. Florentiev, and A. D. Mirzabekov. 1991. Hybridization of DNA with oligonucleotide immobilized in a gel: a convenient method for recording single base replacements. *Mol. Biol. (Mosk.).* 25:718–730.

Livshits, M. A., V. L. Florentiev, and A. D. Mirzabekov. 1994. Dissociation of duplexes formed by hybridization of DNA with gel-immobilized oligonucleotides. *J. Biomol. Struct. Dyn.* 11:783–795.

Lunney, J., A. Chrambach, and D. Rodbard. 1971. Factor affecting resolution, band width, number of theoretical plates, and apparent diffusion coefficients in polyacrylamide gel electrophoresis. *Anal. Biochem.* 40: 158–173.

Maher, L. J., III, P. B. Dervan, and B. J. Wold. 1990. Kinetic analysis of oligodeoxyribonucleotide-directed triple-helix formation on DNA. *Biochemistry.* 29:8820–8826.

Olivas, W. M., and L. J. Maher, III. 1994. DNA recognition by alternate strand triple helix formation: affinities of oligonucleotides for a site in human p53 gene. *Biochemistry.* 33:983–991.

Revzin, A., J. A. Ceglarek, and M. M. Garner. 1986. Comparison of nucleic acid-protein interactions in solution and in polyacrylamide gels. *Anal. Biochem.* 153:172–177.

Rougee, M., B. Faucon, J.-L. Mergny, F. Barcelo, C. Giovannangeli, T. Garestier, and C. Helene. 1992. Kinetics and thermodynamics of triple-helix formation: effects of ionic strength and mismatches. *Biochemistry.* 31:9269–9278.

Wetmur, J. D. 1991. DNA probes: applications of the principles of nucleic acid hybridization. *Crit. Rev. Biochem. Mol. Biol.* 26:227–259.