Experimental Expression in Mice and Spontaneous Expression in Human SLE of Polyomavirus T-antigen

A Molecular Basis for Induction of Antibodies to DNA and Eukaryotic Transcription Factors

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Abstract

We have previously demonstrated that experimental expression of the polyomavirus transcription factor T-antigen has the potential to induce anti–DNA antibodies in mice. Two sets of independent evidences are presented here that demonstrate a biological relevance for this model. First, we describe results demonstrating that mice inoculated with T-antigen–expressing plasmids produced antibodies, not only to T-antigen and DNA, but also to the DNA-binding eukaryotic transcription factors TATA-binding protein (TBP), and to the cAMP-response-element-binding protein (CREB). Secondly, we investigated whether polyomavirus reactivation occurs in SLE patients, and whether antibodies to T-antigen, DNA, and to TBP and CREB are linked to such events. Both within and among these SLE patients, frequent polyomavirus reactivations were observed that could not be explained by certain rearrangements of the noncoding control regions, nor by corticosteroid treatment. Linked to these events, antibodies to T-antigen, DNA, TBP, and CREB were detected, identical to what we observed in mice. Antibodies recognizing double-stranded DNA were confined to patients with frequent polyomavirus reactivations. The results described here indicate that cognate interaction of B cells recognizing DNA or DNA-associated proteins and T cells recognizing T antigen had taken place as a consequence of complex formation between T ag and DNA in vivo in the context of polyomavirus reactivations. (*J. Clin. Invest.* 1997. 99:2045–2054.) Key words: antiDNA • polyomavirus • noncoding control region • T antigen • SLE

Introduction

Antibodies against mammalian native B-form dsDNA serve as diagnostic markers for SLE (1, 2), and subgroups within this antibody population may induce kidney disease typical for SLE (3, 4). Genetic studies strongly indicate that anti–DNA antibodies in SLE derive from an antigen-selective stimulus (5, 6). The antibodies are oligoclonal, somatically mutated, and their V regions contain structures that are selected by, and favor binding to, dsDNA (7, 8). Highly similar SLE-related anti–DNA antibodies (with regard to specificity and structure of their V-regions) can be experimentally induced by artificial DNA–polypeptide complexes (9–11).

During recent years, we developed an experimental model that allowed us to investigate the role and origin of one DNA-binding protein as a molecular basis for rendering DNA immunogenic. In a series of experiments using intact polyomavirus particles or viral DNA–protein complexes as immunogens, all animals responded by producing anti–DNA antibodies, some of which also bound to the supercoiled kinetoplast DNA of *Crithidia luciliae* (12), demonstrating a specificity for dsDNA (for review see reference 13). Recently, we presented evidence that a linked immune response to ssDNA, dsDNA, and histones, relied on the DNA-binding property of the virus-encoded T-antigen transcription factor (T-ag)[1] (14). The experimental systems described so far have, therefore, provided insight into the hitherto unresolved problem of how DNA may be rendered immunogenic.

The structural basis for the development of such antibodies in vivo has, however, not been identified. The experimental polyomavirus model for induction of antibodies to DNA and dsDNA may also prove useful in this context. The majority of human individuals harbor silent polyomavirus infections for their lifetimes (15, 16). Normally, virus reactivations are rare. Little is known about the cell tropism for polyomaviruses, but the kidneys seem to be the major focus, both for primary infection, and also for reactivations (17). During such events, virus particles are shedded in the urine. Performing PCR using primers for viral DNA has therefore been widely used for detecting polyomavirus reactivations. Thus, consecutive urine samples from humans may be assayed for viral DNA shedding, and serial serum samples may be assayed for development of anti–T-ag antibodies, reflecting the expression of the viral transcription factor T-ag. The simultaneous presence of viral DNA sequences in urine samples and anti-T-ag antibodies in serum samples of individual patients may reflect virus reactivation.

In the present report, we performed two lines of experiments that demonstrate a central role for polyomavirus T-ag to give immunogenic potential to nucleosomal antigens. In a first set of experiments, we inoculated Balb/C mice (*a*) with

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1. *Abbreviations used in this paper:* CT, calf thymus; CREB, cAMP response-element-binding protein; NCCR, noncoding control region; RF, rheumatoid factor; T-ag, T-antigen; TBP, TATA-binding protein.

plasmids encoding T-ag under the control of constitutive eukaryotic promoters, and (*b*) with control plasmids. Previous similar experiments (14) have demonstrated the potential for T-ag to initiate the production of antibodies to T-ag, DNA, dsDNA, and histone antigens. Here, we confirm these results, and extend the observations by including the detection of antibodies to the DNA-binding eukaryotic transcription factors TBP (TATA-binding protein) and CREB (cAMP response-element-binding protein) (18, 19). These are novel, not previously described autoantibodies.

In a second set of observations, we have taken advantage of the fact that virtually all individuals are latently infected by polyomaviruses. This allows us to investigate whether polyomavirus reactivation, initiated by active expression of the T-ag, is sufficient for initiating antibody production against the same set of autoantibodies as those detected in mice inoculated with T-ag–expressing plasmids.

In agreement with observations in mice inoculated with T-ag–expressing plasmids, antibodies to ssDNA, dsDNA, TBP, and CREB were, with one exception, confined to individuals demonstrating polyomavirus reactivations, as determined by PCR analyses of serial urine samples, and, linked to such events, by development of anti–T-ag antibodies. By combining these independent results, we may now be able to envisage the following model representing one natural molecular bases for induction of SLE-related anti–DNA antibodies. Silent (latent) polyomavirus infection may be terminated by expression of T-ag. This termination may in turn result in the generation of complexes between T-ag and DNA, rendering DNA and DNA-associated self proteins like TBP and CREB immunogenic.

Methods

Mice. 2-mo–old Balb/C mice were obtained from the Charles River Breeding Laboratories (Sultzfeld, Germany), and were housed in the facilities of the Animal Research Department at the Institute of Medical Biology, University of Tromsø, Tromsø, Norway.

Plasmids. The plasmids pRcCMV-BLT, pRcCMV-SLT, pRcCMV-$SLT_{155T\rightarrow S}$, and pBS-BLT have been characterized in detail elsewhere (14).

Inoculation of plasmids. Groups of five Balb/C mice were injected with a total of 100 μg of the individual plasmids per animal in both lumbar regions. Sera were subsequently collected every second week over an 8-wk observation period, and were analyzed as described below.

Patients. 10 normal individuals and 6 RA patients were followed over 3 mo. 20 SLE patients, all fulfilling four or more of the American Rheumatism Association (ARA) classification criteria for SLE (20), participated in this study over approximately 1 yr. The normal individuals and RA patients were related to the SLE patients with regard to race (Caucasians), sex, and age.

Serum and urine samples. Serum samples, collected at the beginning, and monthly thereafter from each normal individual, RA, or SLE patient throughout the observation period, were stored at −70°C until they were analyzed for antibodies to T-ag, calf thymus (CT) ssDNA, CT dsDNA, TBP, CREB, and rheumatoid factor (RF). Urine samples (20 ml) (approximately one every second week if not otherwise stated in Table II) were frozen by the patients at home and, upon arrival in the hospital, were stored at −70°C until they were examined by PCR. From 32 normal and 36 RA patients, single urine samples were also collected.

PCR amplification of polyomavirus DNA sequences in urine samples. All PCR tests were performed following the guidelines cited by Kwok (21). Preparation of the urine samples before PCR analyses has been described (22). For the detection of polyomavirus early gene sequences, the PYV.for and PYV.rev primers were used (23). To amplify viral NCCR sequences, a nested PCR was performed. Initially, 25 cycles were run with the primer set GPPY-1 and GPPY-2, followed by 30 cycles with the BKTT-1 and BKTT-10 primers (24, 25). Both primer sets are flanking the viral NCCR. Under these conditions, 0.1 fg of polyomavirus early gene sequence could be detected (17 genome equivalents). To control negative PCR test results, a parallel sample of each PCR-negative urine specimen was amplified with the addition of 1 fg of BK virus DNA. Amplified bands of expected size allowed the conclusion that PCR-negative urine samples are truly negative. To distinguish between polyomavirus BK or JC NCCR, PCR products were digested with PflmI, Bsu36I and SacI (24–26).

Sequencing the NCCR. Amplified NCCRs were sequenced twice by the dideoxy method (27) as described in detail elsewhere (22).

Antigens. The source and preparation of T-ag, CT ssDNA, and CT dsDNA has been described previously (14, 28). The transcription factors TBP and CREB were obtained from Santa Cruz Biotechnology, Inc. (San Diego, CA). TBP is a full-length human protein prepared as a glutathione-*S*-transferase (GST) fusion protein in *Escherichia coli*, while CREB is a 10–14-kD DNA-binding domain (CREB-1) of the 43-kD full-length human protein. TBP and CREB were > 98% pure according to the manufacturers, and were virtually free from contaminating proteins according to SDS-PAGE (data not shown).

Indirect solid phase ELISA. For detection of antibodies to CT ssDNA, CT dsDNA, and T-ag, the ELISA tests were performed as described previously (14, 28). The CT ssDNA used as ELISA antigen binds both anti–ssDNA and anti-dsDNA antibodies. In the CT dsDNA ELISA, specificity for dsDNA was ascertained by the absence of binding of the monoclonal anti–ssDNA antibody 111-185 (29), while the monoclonal anti–dsDNA antibody DNA6 (8) bound to this antigen. For detection of antibodies to TBP and CREB, microtiter plates (Nunc Maxisorp; Intermed Nunc, Copenhagen, Denmark) were coated with TBP or CREB at a concentration of 1 μg/ml in PBS. For these tests, serum samples were diluted twofold from 1/100 to 1/

Table I. Demonstration of Antigen-Selective Immune Responses to T-ag and to Various Nucleosome-associated Autoantigens in BALB/c Mice Inoculated with T-ag-expressing and Control Plasmids

Plasmid	Protein expressed	Balb/C	Mean titer of antibodies to				
		n	T-antigen	ssDNA	TBP	CREB	RF
pRcCMV-BLT	BK virus T-ag	5	248±48	352±97	468±254	552±186	0
pRcCMV-SLT	SV40-$T\text{-}ag_{155T}$	5	314±118	350±138	318±99	402±91	0
pRcCMV-$SLT_{155T\rightarrow S}$	SV40-$T\text{-}ag_{155S}$	5	282±28	< 100*	346±115	74±68	0
pBS-BLT	None	5	0 -	0 -	0 -	0 -	0

*In sera from these mice, traces of anti–DNA antibodies could be detected (see Fig. 1 *C*), but the OD (492) values were below the binding value at 1/100 dilution necessary to reach a titer.

3200 in PBS (0.05% Tween-20). To ascertain that serum antibodies actually bound to TBP, and not to its fusion partner GST, the sera were tested in control wells coated with GST in PBS at a concentration of 1 μg/ml. These ELISAs were processed exactly as described for the other tests. For all ELISA tests, only Ig antibodies were analyzed, using a horseradish peroxidase-conjugated goat anti-human Ig. Titers of the antibodies were determined as described previously (14). The ELISA tests were processed using the fully automated Behring ELISA processor III (Behringwerke AG, Frankfurt a. Main, Germany).

Crithidia luciliae. The test for detection of antibodies to dsDNA was performed as described elsewhere (12).

Detection of RF. This was done by a nephelometric method using a Nephelometer-Analyzer (Behring, Marburg, Germany). The results are given as IU/ml, using the reference standards supplied by the manufacturer. Values ≤ 11 IU/ml are regarded as negative.

Statistics. Statistical testing was done using the two-way analysis of variance with patients' number, group (I–IV), and multiple regression analyses as factors. SAS software was used (SAS Institute, Inc., Cary, NC).

Results

T-ag expression in plasmid-inoculated mice. Groups of five mice were given a single injection of plasmids encoding wild-type T-ag from polyomavirus BK (pRcCMV-BLT), SV40 virus (pRcCMV-SLT), or the mutant SV40 T-ag (pRcCMV-$SLT_{155T\rightarrow S}$). This latter mutant T-ag, due to replacement of one amino acid at position 155, has lost its ability to bind DNA sequence-specifically, but has retained a weak, sequence nonspecific affinity for DNA (30). Sera collected every second week over an 8-wk period, were assayed for Ig antibodies to T-ag. While all preimmune sera from these mice were negative for anti–T-ag antibodies, this antibody increased over the observation time (Fig. 1, *A–C*), and sera drawn after 8 wk contained high titers of anti–T-ag antibodies, indicating that T-ag was expressed in vivo (Table I). Sera from mice inoculated with the plasmid containing the gene encoding BK virus T-ag, but lacking an eukaryotic promoter (pBS-BLT), were negative for anti–T-ag antibodies (Fig. 1 *A*).

T-ag expression and initiation of antibodies to DNA and the transcription factors TBP and CREB. The mice that received the plasmids expressing wild-type T-ag from BK or SV40 virus, but not those inoculated with the pBS-BLT plasmid, developed Ig antibodies to ssDNA, TBP, and CREB simultaneously with the initiation of anti–T-ag antibodies (Table I, and Fig. 1, *A–B*). In mice injected with the plasmid encoding the mutant T-ag, antibodies to T-ag and to TBP developed in a similar way (Table I, Fig. 1 *C*). However, in only three out of the five mice, anti–CREB antibodies were detected at titers > 100 (Table I). In the two other mice, weak reactivity to CREB was also detected (Fig. 1 *C*), but the strength was below the level necessary to reach a titer at a 1/100 serum dilution. None of these mice produced anti–DNA antibodies above this threshold, although weak reactivities could also be detected for this specificity (Fig. 1 *C*) in agreement with previous results (14). Antibodies to IgG (RF) were not detected in any of these sera, indicating that the responses to the other antigens are the result of antigen-selective B cell stimulation.

Correlation between T-ag expression in vivo and immune responses to DNA, dsDNA, TBP, and CREB in humans. As indicated above, PCR-based detection of polyomavirus DNA sequences in urine samples, combined with development of anti–T-ag antibodies, are regarded as evidence for polyomavirus reactivation. These parameters were therefore selected to investigate whether the processes resulting in antibodies to T-ag, DNA, TBP, and CREB after experimental T-ag expression in mice reflect similar processes in humans, particularly in those with SLE.

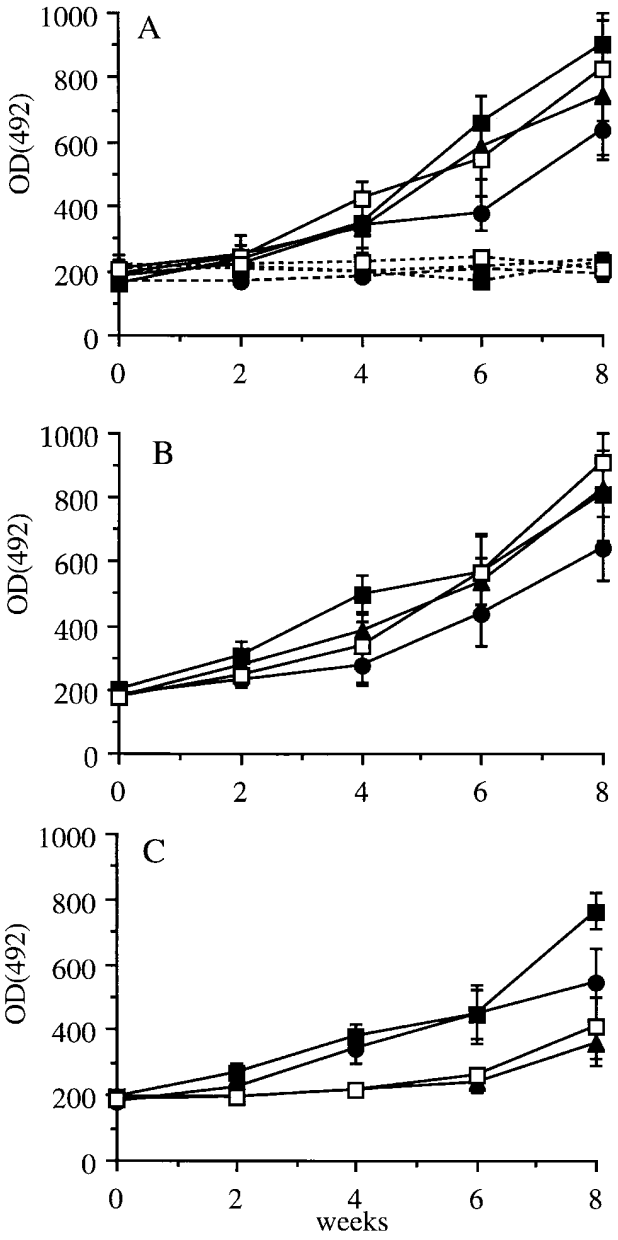

Figure 1. Immune responses to T-ag (■), DNA (▲), TBP (●) and CREB (□) in groups of mice given a single injection of the T-ag–encoding plasmids pRcCMV-BLT (*A*, *solid lines*), pBS-BLT (*A*, *broken lines*), pRcCMV-SLT (*B*), or pRcCMV-$SLT_{155T\rightarrow S}$ (*C*). Results are given as mean±SD of the absorbance at 492 nm, representing antibody binding at serum dilution 1/100 for all tests.

Prevalence and frequency of human polyomavirus DNA sequences in urine samples from healthy individuals and RA patients. Single urine samples from 32 healthy and 36 RA individuals were analyzed. Except for one urine sample taken from an RA patient, polyomavirus DNA sequences were not

Table II. PCR-amplified Polyomavirus DNA Sequences in Urine Samples, and Mean Anti-T-ag and Anti–ssDNA Antibody Titers, as Determined in 20 SLE Patients Analyzed Over 1 yr

		PCR positive			Anti-T-ag titer*			Anti-DNA titer*		
Group	Patient	*n*	*n*	%	Mean	SD	CV	Mean	SD	CV
I	7	16	0	0	0.0	–	–	0.0	–	–
	11	18	0	0	0.0	–	–	0.0	–	–
	19	20	0	0	0.0	–	–	758.0	101.41	13.38
	21	19	0	0	0.0	–	–	0.0	–	–
II	9	18	1	6	41.0	86.47	210.90	21.0	44.33	211.12
	13	27	1	4	31.8	54.74	172.04	21.8	48.54	222.49
III	2	22	2	9	26.7	53.15	199.32	11.1	33.33	300.00
	4	33	7	21	217.5	44.75	20.57	146.2	35.36	24.18
IV	5	28	24	86	854.6	109.12	12.77	956.4	277.25	28.99
	8	21	12	63	458.0	24.86	5.43	341.0	121.33	35.58
	10	32	16	50	315.5	105.20	33.35	301.8	113.91	37.74
	12	23	23	100	475.0	39.23	8.26	801.0	126.35	15.77
	14	6	6	100	556.6	76.33	13.71	725.0	66.26	9.14
	16	28	25	89	484.5	67.29	13.89	496.8	77.27	15.55
	17	27	21	78	454.5	42.75	9.49	466.4	158.76	34.04
	18	23	11	48	467.8	96.92	20.72	206.7	76.16	36.85
	20	20	14	70	777.0	164.99	21.43	788.0	256.51	32.55
	23	6	6	100	471.3	25.32	5.37	567.5	90.36	15.92
	24	19	15	79	686.7	77.46	11.28	631.1	155.28	24.60
	25	23	7	30	432.2	64.76	14.98	532.2	83.48	15.69

*The titers of antibodies to T-ag and to ssDNA are presented as mean of all titers measured over the observation time for each patient.

detected in any of these samples (data not shown), while 43% of urine samples from SLE patients were PCR-positive (see below). For the 10 normal individuals followed over 3 mo, none of the urine samples (six from each patient) contained polyomavirus DNA sequences. None of the four serum samples from each of these individuals contained antibodies to T-ag, DNA, TBP, or CREB. For the six RA patients followed over 3 mo, five were PCR- (as well as serum antibody) negative. For one, however, four out of six urine samples were PCR-positive, and all serum samples from this patient contained antibodies to T-ag, ssDNA, TBP, and CREB, with mean titers of 219, 147, 312, and 289, respectively (data not shown).

Frequency and NCCR structure of polyomavirus DNA in serial urine samples from SLE patients. For all PCR-positive urine samples, amplicons were obtained with the primer set complementary to T-ag–encoding DNA sequences and to sequences flanking NCCR. Based on the frequency of urine samples containing polyomavirus DNA sequences, the patients were divided into four groups (Table II). Group I encompassed patients 7, 11, 19, and 21, whose urine samples were negative for polyomavirus DNA throughout the observation period. In group II (patients 9 and 13), one single PCR-positive urine sample was identified for each of the patients. Patients demonstrating few PCR-positive urine samples were clustered in group III, with patient 2 having two PCR-positive urine samples out of 22 (9%), and patient 4 having seven out of 33 (21%). About 30–100% of the urine samples of the remaining 12 patients (group IV) were PCR-positive (Table II). Of the total number of urine samples in this group, 74% were PCR-positive, compared to 43% for all SLE patients. The frequency and distribution of PCR-positive urine samples of patients representative for groups I–IV are depicted in Fig. 2.

The high prevalence of polyomavirus DNA in the urine of SLE patients could theoretically be explained by the selection of virus strains with certain NCCRs known to dispose for reactivation (15, 31). Duplication of P-blocks (31) of BK virus NCCR have been particularly ascribed this effect. The sequences of these NCCRs (Table III), however, were almost identical to those of the archetypal polyomavirus BK (31) or JC (24), in agreement with restriction enzyme patterns of the amplified NCCR (data not shown). These sequences except the one in patient 2, that contained an unreported BK virus NCCR (denoted AO), are all found among normal individuals.

Corticosteroid treatment and polyomavirus reactivations. As the strong tendency for virus reactivation could not be explained by selection of certain transcriptional active NCCRs, treatment of the patients with steroid hormones, which have been shown in vitro to increase the permissivity of cells for BK virus (32), could alternatively account for the enhanced productive polyomavirus infection among these patients. This can not, however, be the explanation for the strong tendency for polyomavirus reactivations, as SLE patients 8, 12, 14, 23, and 24, all with frequent reactivations, did not receive corticosteroids during the observation period.

Table III. Anatomy of the Noncoding Control Region (NCCR) of the Polyomaviruses BK or JC Detected in the Urine Samples of SLE patients. The NCCRs Were Compared to the Consensus Sequence of the Archetypal BK Virus WW or the JC Virus CY Strains (24, 31)

Virus	Patients*	Strain	NCCR anatomy
BK	4, 10, 13, 18, 25	Consensus WW	$P_{1\text{-}68}$-$Q_{1\text{-}39}$-$R_{1\text{-}63}$[§]
	5,[‡] 8,[‡] 24,[‡] 16,[‡] 17,[‡] 20[‡]	WW	$P_{1\text{-}68}$-$Q_{1\text{-}39}$-$R_{1\text{-}63}$
	2	AO	$P_{1\text{-}68}$-$Q_{1\text{-}39}$-$R_{4\text{-}9}$-$P_{22\text{-}68}$-$Q_{1\text{-}39}$-$R_{1\text{-}63}$
JC	12	Consensus CY	25bp-23bp-55bp-66bp-18bp-69bp[¶]
	14,[‡] 23[‡]	CY	25bp-23bp-55bp-66bp-18bp-69bp

*From patient 9, no sequence was obtained. [‡]For these patients, the NCCRs contained point mutations compared to the consensus sequence of BKV (WW) or JCV (CY), respectively (A. Sundsfjord et al., manuscript in preparation). [§]The BKV NCCR is divided into P-, Q-, and R-blocks. They consist of 68, 39, and 63 base pairs, respectively. The lower case numbers represent the borders of each block. [¶]The JCV NCCR is divided into blocks according to the number of base pairs.

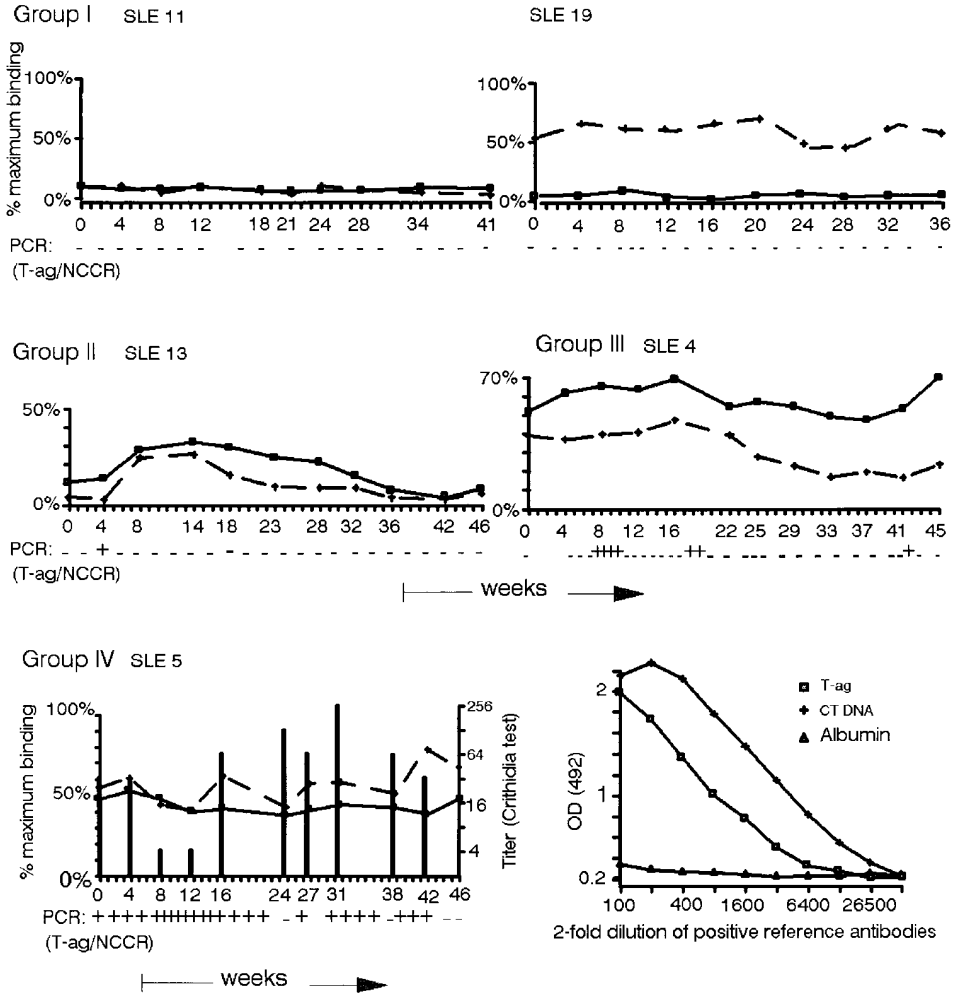

Figure 2. Polyomavirus reactivation and immune responses to T-ag and DNA in human SLE. Antibody activities against CT DNA (-+-) and polyomavirus T-ag (-■-) at the given time points are plotted against results from PCR-amplified urine samples using primers complementary to NCCR and T-ag–encoding polyomavirus DNA (+: PCR-positive urine samples; −: PCR-negative urine samples). ELISA results are presented at nonsaturating serum dilutions for the individual patient; binding values are given as % of maximum binding of reference antibodies. The ELISA results are given at serum dilution 1/100 for patient 11, 1/200 for patients 13 and 4, 1/500 for patient 19, and 1/1000 for patient 5. For mean titers of the antibodies for the individual patients, see Table II. The twofold dilution curves for the reference serum used for determination of titers of the individual antibodies is presented in the lower right panel. The bars in the figure for patient 5 represent titers in the *Crithidia luciliae* test for this patient.

Anti–T-ag antibodies related to detection of viral DNA sequences in urine samples from SLE. Detection of polyomavirus DNA corresponding to T-ag encoding sequences by PCR did not prove the expression of large T-ag, which is considered essential for rendering DNA immunogenic (14). We therefore tested serial serum samples from each SLE patient for the presence of anti–T-ag antibodies, an indication of T-ag expression.

These antibodies were not detected in any serum sample from patients in group I (Table II, Fig. 2 for patients 11 and 19). In sera of patients of group II, weak and transient anti–T-ag antibody response was detected in patient 13 (Table II, Fig. 2), preceded by the single episode of virus reactivation. A similar result was also obtained for patient 9, although critical serum samples were missing for this patient. The last negative sample (drawn at week 16), however, preceding the PCR-positive serum sample (week 22), was followed by a serum sample at week 28 positive for anti–T-ag antibodies (data not shown). The course of this response could not be determined for this patient as the single PCR-positive urine sample appeared near the end of the observation time. Thus, in patient 13, and also possibly for patient 9, we could directly determine an anti–T-ag antibody response preceded by a single episode of polyomavirus reactivation.

Both patients in group III had similar anti–T-ag responses (Table II, Fig. 2 for patient 4) connected in time to the PCR-positive urine samples. For patient 4, the anti-T-ag response fluctuated above the baseline (mean titer for the observation period was 217), and the titer increased following episodes of viral DNA shedding. A similar pattern, however less distinct because of weak anti–T-ag antibodies was observed for patient 2 (data not shown).

For the remaining 12 patients (group IV), strong and persistent production of antibodies to the T-ag, with mean titers ranging from 315 to 854, was recorded (Table II, Fig. 2 for patient 5).

These data argue for the expression of the T-ag in these patients. In agreement with these data, there was a strong positive correlation between frequencies of PCR-positive urine samples and mean titers of anti–T-ag antibodies (Fig. 3 *A*, $r = 0.86$, $P < 0.0001$). Likewise, there was an inverse relationship between the frequency of PCR-positive urine samples and coefficient of variance (CV) of the anti-T-ag titers in individual patients (Table II), consistent with the idea that T-ag expression induced this antibody in vivo. Thus, frequent or persistent expression of the T-ag resulted in high and stable titers (as demonstrated by low CV) of the anti–T-ag antibodies, as expected for an antibody induced by an acquired nonself antigen like the T-ag.

The relationship between polyomavirus DNA shedding, anti–T-ag antibodies, and development of anti–DNA antibodies in SLE patients. In group I, anti–DNA antibodies could not be detected for patients 7, 11 (Fig. 2 *A*), and 21 (Table II). For

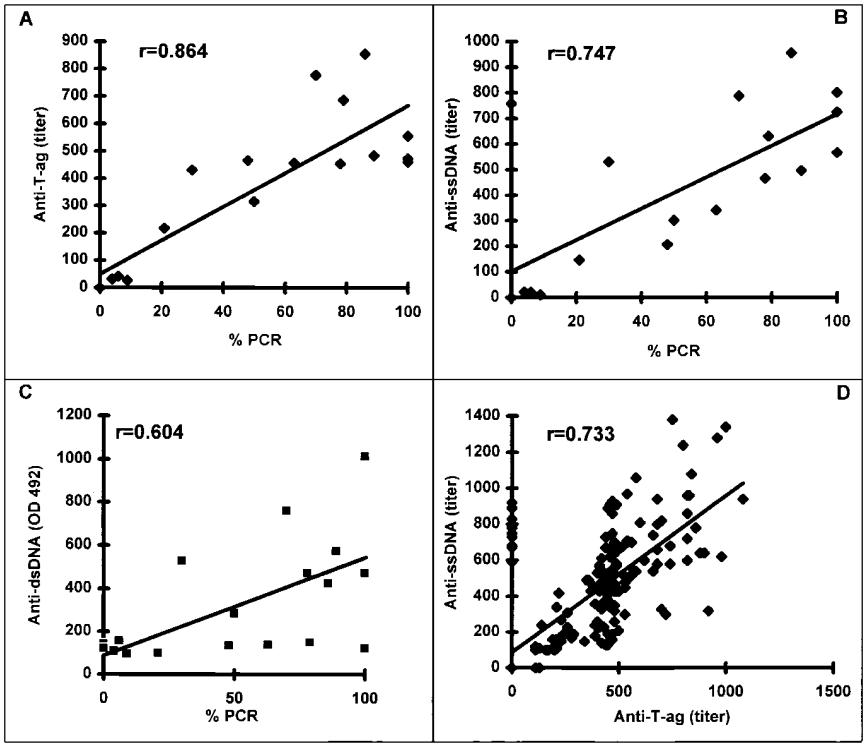

Figure 3. Anti-T-ag and anti–DNA antibody titers and their correlations with frequency of polyomavirus reactivations in human SLE. Mean of anti-T-ag titer (*A*), anti-ssDNA titer (*B*), OD 492 of serum antibody binding to CT dsDNA at a 1/100 dilution (cutoff value was 0.2 OD units) (*C*) over the observation time plotted against the frequency of PCR amplified urine samples for each individual patient. In *D*, correlation between anti-T-ag and anti–DNA antibody titers at all time points for all patients are given. The correlation coefficient (r) is indicated in each figure.

patient 19, however, strong and persistent anti-ssDNA response was observed, with titers ranging from 590 to 920, mean titer 758 (Table II, Fig. 2).

For both patients in group II, antibodies to ssDNA were detected (Table II). In patient 13, the virus reactivation, as determined by the combined detection of single PCR-positive urine samples and subsequent development of antibodies to T-ag, preceded the transient production of antibodies to DNA (Fig. 2). Similarly, in patient 9, antibodies to ssDNA increased simultaneously with antibodies to T-ag after the single PCR-positive urine sample appearing near the end of the observation period. The course of this response could not be determined.

Similar results were obtained for patients 2 and 4 in group III (Table II). Also, for these patients, production of antibodies to ssDNA were related in time to PCR-positive urine samples, and to the development of antibodies to T-ag with mean antiDNA titers of 11 (ranging from 0–100) and 146 (ranging from 100–190) for patients 2 and 4, respectively (Table II, Fig. 2, patient 4).

For patients in group IV, anti–ssDNA antibodies persisted at high levels throughout the observation period, with mean titers ranging from 207 to 956 (Table II), similar to those observed for anti–T-ag antibodies. Patients 5, 12, 14, and 20 produced anti–*ds*DNA antibodies detectable by the *Crithidia luciliae* assay, with mean Crithidia titers of 64, 33, 20, and 67, respectively, over the observation time (Fig. 2 for patient 5). These, and three additional patients (16, 17, and 25) in group IV produced anti–dsDNA antibodies detectable in an ELISA specific for dsDNA (Fig. 3 *C*). Thus, except for patient 19, the presence of anti–DNA antibodies was limited to patients shedding polyomavirus DNA sequences in their urine, while antibodies with specificity for dsDNA were restricted to group IV patients.

As for anti–T-ag antibodies, the mean anti-ssDNA titers correlated strongly with the frequency of PCR-positive urine samples (Fig. 3 *B*, $r = 0.747$, $P < 0.0001$). An inverse correlation between this frequency and CV of the mean anti-ssDNA titers was also established (Table II), indicating that recurrent PCR-positive urine samples within single individuals of group IV reflect recurrent stimulation of the immune system consistent with high and stable anti–DNA antibody titers. This, and the fact that there was a strong positive correlation between anti–T-ag and anti–ssDNA antibody titers in all patients at all time points (Fig. 3 *D*, $r = 0.733$, $P < 0.0001$), strongly indicate that anti–DNA antibodies, as antibodies to the T-ag, are induced as a consequence of T-ag expression in vivo. The strength of anti-dsDNA also increased with increasing frequency of polyomavirus reactivation (Fig. 3 *C*, $r = 0.60$), and there was a significant correlation between anti–dsDNA antibodies and their presence in group IV patients ($\chi^2 = 7.18$, $P < 0.01$).

The presence of RF in these patients did not correlate with polyomavirus reactivations, nor with presence of anti–T-ag antibodies from the following observations. Only four of the 20 patients produced this autoantibody (patient 21 in group I, and patients 8, 16 and 23 in group IV). Thus, in 9 out of the 12 patients in group IV, and in all patients belonging to group II or III, RF was not detected. Among 112 other sera from autoimmune patients (RA, Sjøgren syndrome, and SLE), 39 contained antibodies to T-ag. Of these, 8 (21%) were positive for RF, while 17 out of the 73 anti-T-ag negative sera (23%) contained RF ($r = 0.035$, $P > 0.5$, data not shown).

Antibodies to TBP and CREB. Because of small available amounts of TBP and CREB molecules, only sera with the highest levels of anti-T-ag and anti-DNA activities, together with randomly selected sera from SLE group I, were tested for antibodies against the transcription factors TBP and CREB.

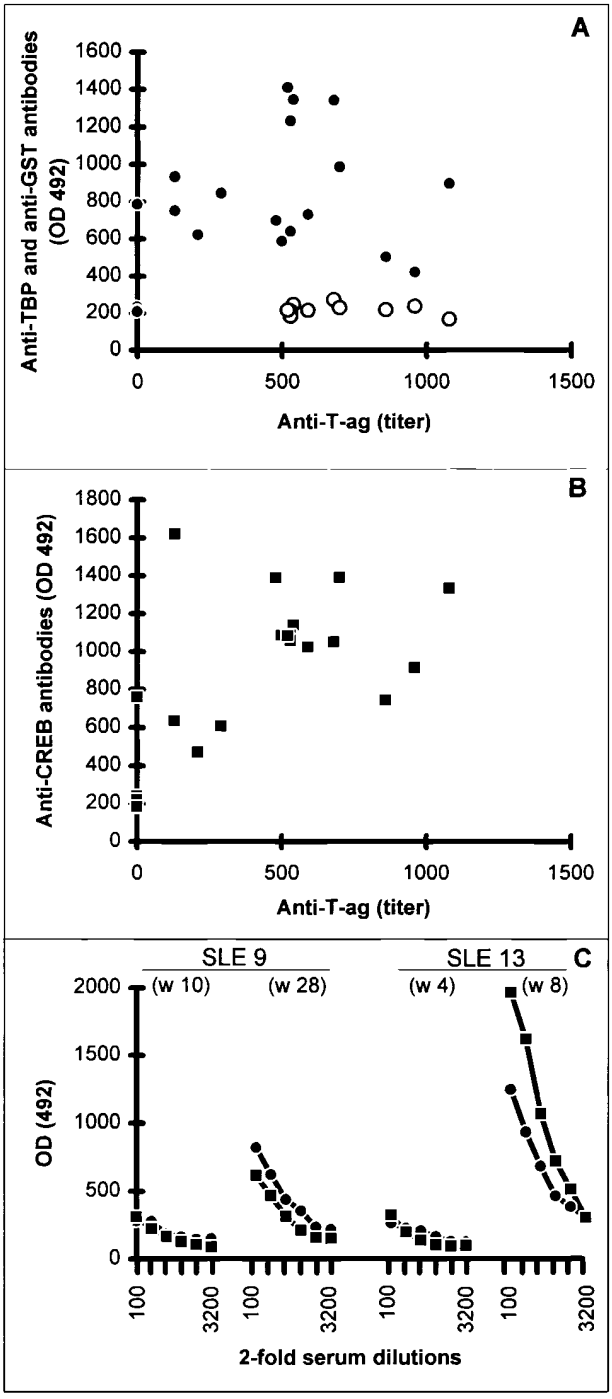

Figure 4. Serum antibodies to TBP (●) and GST (○), (*A*) and CREB (■, *B*) are plotted against titers of anti–T-ag antibodies in selected sera from individual SLE patients. The binding values (OD 492) for antibodies to TBP and CREB are given at nonsaturating (i.e., 1/200) dilutions of the sera. For patients 9 and 13 (group II), serum antibodies to TBP (●) and CREB (■) before and after the single polyomavirus reactivations are demonstrated. Binding values are given as OD 492 at twofold serum dilutions from 1/100 to 1/3200.

As demonstrated in Fig. 4, all anti-T-ag positive sera tested from groups II–IV (in addition to the serum from patient 19 of group I) were positive for anti–TBP (Fig. 4 *A*) and anti–CREB (Fig. 4 *B*) antibodies. In sera from group II patients (9 and 13) drawn before the virus expression episode (week 10 and week 4 for patients 9 and 13, respectively), no or weak anti–TBP and anti–CREB antibodies were detected. Sera drawn after this time point (week 28 and 8, respectively), however, were increasingly positive in these tests (Fig. 4 *C*). These results strongly indicate that antibodies to TBP and CREB, like antibodies to DNA, were initiated as a consequence of T-ag expression. This is consistent with the observation that all preimmune sera from plasmid-inoculated mice, and all sera of group II patients drawn before virus reactivations, were negative in the TBP and CREB ELISAs. Not all sera contained antibodies to TBP and CREB (e.g., sera from patients 7, 11, 21 of group I), and 10 sera from group IV patients positive for such antibodies did not bind in an ELISA specific for GST (Fig. 4 *A*). These results argue against binding of serum antibodies to bacterial proteins contaminating the TBP and CREB preparations, since this GST is also purified from *E. coli.*

Discussion

In the present report we have investigated whether polyomaviruses have, in a natural situation, an ability to induce antibodies to DNA and DNA-associated self proteins similar to that previously observed in an experimental context in rabbits or mice (11–14, 28, 33). For these studies, we have taken advantage of the fact that virtually all human individuals are latently infected by such viruses. The biological requirement for polyomaviruses to reactivate is fulfilled upon in vivo expression of the viral transcription factor T-ag.

Recently, we have demonstrated that expression of T-ag is sufficient to initiate the production of antibodies to DNA (14). Here, we have extended the observations on the immune responses to T-ag expression in mice to include detection of antibodies, not only to DNA, but also to the transcription factors TBP and CREB. The TBP and TBP-associated factors form the multiprotein complex TFIID, which is part of the transcription preinitiation complex. TBP is the only component of TFIID that binds directly to the TATA box of DNA (18). TBP is expressed in all cell types (18). In vitro studies have also demonstrated that TBP can interact with SV40 T-ag by direct protein–protein interaction (34). CREB proteins, on the other hand, belong to a large family of related proteins which also includes the CREMs (cAMP-response element modulators) and the ATFs (activating transcription factors). Genes whose expression are regulated by the secondary messenger cAMP often contain the palindromic consensus sequence GTGACGTCA, the so-called cAMP response element (CRE). The CRE binding protein (CREB) binds constitutively to this sequence (19). It is not known whether CREB can bind T-ag. Thus, both TBP and CREB bind directly to DNA or nucleosomes (18, 34). It was therefore tempting to speculate that if T-ag binds DNA or nucleosomes (35), not only DNA and histones (14), but also DNA-associated polypeptides like TBP and CREB are rendered immunogenic as a consequence of T-ag expression and subsequent binding to DNA.

In mice inoculated with the plasmids encoding wild-type T-ags, strong immune responses to T-ag, DNA, TBP, and CREB were detected. Sera from mice inoculated with the mutant T-ag-expressing plasmid contained strong antibodies to T-ag and to TBP, while the responses to DNA and CREB were less pronounced. This mutant T-ag binds only weakly in a sequence-unspecific way to DNA, which may explain the weak anti-ssDNA and anti-CREB responses in these mice (Fig. 1 *C*).

These results contrast strikingly with the results for induction of anti–T-ag and anti–TBP antibodies obtained in the same mice, indicating that the mutant T-ag and TBP form a stable complex in vivo resulting in cognate interaction of TBP specific B-cells and T-ag specific T-cells.

These observations represented the rational bases for the present investigations designed to examine the extent of polyomavirus reactivations, and particularly T-ag expression, in SLE, and whether such reactivations reflect a natural biological origin for initiation of antibodies to DNA and to other nucleosome-associated antigens.

A prominent observation made was that of the high frequency of polyomavirus reactivations within and between SLE patients. The NCCRs detected (all except that for patient 2) belonged to the archetypal WW or CY of polyomavirus BK or JC, respectively, found among normal individuals. Neither certain NCCR rearrangements nor administration of steroid hormones to the SLE patients (see results) could therefore explain the strong tendency for virus reactivations in these patients.

The linkage between viral DNA shedding and development of anti–T-ag antibodies strongly indicated that T-ag expression had taken place. The anti–T-ag antibody profiles in individual SLE patients are thus representative for conventional immune responses to a foreign antigen, providing the ideal situation to compare directly a conventional and an assumed autoimmune anti-DNA response, both induced by the same nucleosome-T-ag complex (14). Analyzing the production of anti–DNA antibodies in individual patients demonstrated the validity of this approach. Both the time points for their initiation and their profiles strongly indicated that anti-T-ag and anti–DNA antibody populations were temporally linked to each other and to polyomavirus reactivations for these patients. For group IV patients, anti-T-ag and anti–ssDNA antibodies were produced persistently and at significantly higher levels than for group II and group III patients ($P < 0.05$). Antibodies to dsDNA were exclusively detected in group IV patients. Although there were individual variations in titers over the observation time, a significant correlation existed between the production of anti–T-ag and anti–DNA antibodies in all patients at all time points. In group II patients, we could directly demonstrate that the virus reactivation preceded the development of these antibodies. The data presented here therefore indicate a causal relationship between development of anti–ssDNA/dsDNA antibodies and expression of T-ag in context of polyomavirus reactivations in vivo.

Polyomaviruses infect almost 100% of the human population (15, 16). After primary infection, the virus remains silent, and reactivations of polyomaviruses seem to be rare events in normal individuals (36–40). Thus, from a total of 341 urine samples from normal individuals, or from individuals before transplantation (taken from references 36–40, and 92 urine samples from this study), polyomavirus DNA sequences were detected in seven (2.1%). Anti–T-ag antibodies have been detected in sera of 11 out of 1,240 cancer patients (0.9%), and in 4 out of 501 normal control sera (0.8%) (41). As antibodies to T-ag are a relatively long-lasting and detectable demonstration of T-ag expression in vivo (see Fig. 2, patient 13), these results also indicate that reactivations are rather rare both among and within normal individuals. The opposite result is observed in HIV-infected individuals (22, 42, unpublished observations). Interestingly, among such patients anti–dsDNA antibodies are detected by ELISA (43) or by the Crithidia test (unpublished observations). Polyomavirus reactivations may therefore explain development of anti–DNA antibodies generally, and not exclusively for SLE patients. This is also demonstrated in the present report for one out of six RA patients.

Recurrent expression of polyomaviruses, typical for SLE, may for the following reasons also form the structural bases for the progressive generation of antibodies to dsDNA. The specificity for dsDNA is determined by structural properties of the V-region of the Ig heavy chain, including arginines in the complementary determining region (CDR) III region (5, 7, 44). In autoimmune mice, such structures seem to result from progressive development of an early ssDNA antibody repertoire to include specificity for dsDNA (29). Thus, antibodies to ssDNA may serve as precursor antibodies for anti–dsDNA antibodies. Compatible results have been obtained after extensive immunizations with CT DNA complexed with Fus1 (9), or with polyomavirus BK (11). Intermittent or persistent T-ag expression in man may be a natural parallel to such experimental immunizations, creating the molecular bases for a continuous stimulation of B cells specific for DNA. Through somatic mutations resulting in accumulation of structures predisposing for dsDNA binding (like arginines in the CDR III of the Ig heavy chain), such antibodies may also obtain specificity for dsDNA (7, 44). From this observation, one may predict that the processes responsible for initiation of antibodies to dsDNA may be the same as that responsible for initiation of antibodies to ssDNA. Alternatively, continuous stimulation by DNA in an immunogenic form may also recruit B cells with an inherent specificity for dsDNA because of incomplete deletion of such cells (45). The fact that seven patients, all belonging to group IV, produced anti–dsDNA antibodies is consistent with these interpretations. Sustained or recurrent expression of T-ag and subsequent binding to DNA may represent one molecular basis for such continuous stimulation of B-cells specific for DNA.

That other biologically active processes may be responsible for initiation of anti–DNA antibody responses have not been excluded (see patient 19 of the present study). T cells specific for histones, for example, may both initiate and maintain anti–DNA antibody responses in SLE by processes similar to those described for T-ag, as indicated by recent results from Desai-Mehta et al. (46). These two systems are quite similar, however, with the principal difference being that in the polyomavirus model, T cells recognize a nonself protein (T-ag), while in the system described by Desai-Mehta et al., T cells recognize self proteins contained within the nucleosomes. These two pathways are, however, not mutually exclusive, and may overlap each other within the same patient, or may substitute for each other in different SLE patients.

We cannot entirely rule out that both anti–DNA antibodies and polyomavirus reactivations may independently arise from an underlying yet undefined proximal cause. The consistent results obtained in an experimental context in mice and in a natural context in SLE patients, however, demonstrating a statistically highly significant association between antibodies to T-ag and to DNA, support the view that spontaneous expression of the virus alone represents one basic process for induction of anti–DNA autoantibody production.

The results described in this report are particularly attractive in light of the attempt to understand a biological origin of antibodies to DNA within a natural physiological context. For a latent polyomavirus to reactivate, this process is initiated by

active expression of the T-ag. One consequence of this expression is binding to DNA, necessary for initiating transcriptional activity of virus genes. A side effect is also binding to host DNA, thus forming a complex between host cell DNA and T-ag. This process allows for cognate interaction of B cells specific for either of the components of nucleosomes (i.e., DNA and histones), or for molecules physically linked to this complex (e.g., transcription factors like TBP or CREB), and T-cells specific for T-ag, processed and presented by such B-cells. T-ag may therefore serve as a latent biological basis for anti–DNA antibody production. The results presented here and previously (14) are consistent with this interpretation, and are compatible with the model suggested in a recent review (13) and by Radic and Weigert (5).

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