p53-mediated DNA renaturation can mimic strand exchange

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ABSTRACT

The process of strand exchange is considered to be the hallmark of DNA recombination. Proteins known to carry out such exchange are believed to operate via one or the other of two mechanisms. RecA-like proteins promote the formation of a three-stranded or triplex synaptic intermediate in which strand exchange occurs, whereas other proteins would allow the coordinated exonucleolytic degradation of one strand in the duplex DNA and its replacement by an invading strand of similar sequence and polarity. In view of properties ascribed to it, we have attempted to determine whether p53 belongs to one or the other of these groups of proteins. The in vitro assay used relies on a double-stranded (ds) oligonucleotide (oligo 1+2) and a single-stranded (ss) oligonucleotide (oligo 3), part of which is complementary to oligo 1. The data collected suggest that, under the conditions of the assay, oligo 1+2 undergoes partial denaturation; p53 then catalyzes renaturation of oligo 1 with oligo 3, rather than true strand exchange. Since p53 is not known for being able to ‘melt’ DNA, it would seem unlikely that this protein would effect strand exchange in vivo without assistance from another, denaturing, protein.

INTRODUCTION

In organisms ranging from bacteria to mammals, proteins have been found that are able to promote the kind of interaction between homologous DNA sequences which could be the initial step in DNA repair or recombination (1). The archetype of such proteins is the RecA protein of Escherichia coli (2–9). The best characterized in vitro reaction between RecA and DNA is one in which, in the presence of ATP, RecA first polymerises onto ssDNA; the resulting nucleoprotein filament subsequently forms a joint structure with homologous dsDNA in which the three strands lie parallel; then, pairing occurs between the ssDNA (+)-strand initially present in the filament and the complementary or (−)-strand from the dsDNA; finally, unidirectional branch migration allows complete strand exchange, leading to the release of two DNA products, heteroduplex DNA and the (+)-strand originally included in the dsDNA (2–8). The three-stranded intermediate, which was reported to be stable even after removal of the RecA protein (10–12), is considered to be characteristic of strand exchange catalyzed by proteins of the RecA type (13), a family of proteins which includes the Rad51 (14–16) and Dmc1 (17,18) proteins of Saccharomyces cerevisiae. Other proteins from several eukaryotic species have been shown to carry out strand transfer in vitro. However, these proteins have no sequence similarity to RecA, Rad51 or Dmc1, do not need ATP for carrying out strand exchange, and fail to give rise to a nucleoprotein filament (1,19–23). Yet, they often have exonuclease-associated activity (1) which, in some instances at least, is not only essential for strand exchange but dictates the polarity of the reaction (1,23).

The p53 tumor suppressor (Fig. 1A) is a eukaryotic protein known to be involved in DNA repair, at least indirectly (25,26). This transcriptional activator accumulates in cells in response to exposure to ionizing radiation or other DNA-damaging agents (25,26), and stimulates the activity of the GADD45 (DDT11) gene, which is believed to be involved in the response to DNA damage (27–29). Aside from this indirect involvement in DNA repair, p53 is suspected to play another, more immediate, role in this process. Indeed, several groups have reported that p53 binds single-stranded DNA ends and catalyzes both DNA renaturation (30–34) and strand exchange (31,34), in the absence of ATP. Recently, p53 has also been found to possess exonuclease activity (35). While DNA renaturation can be defined as the establishment of a duplex structure between two complementary ssDNAs, strand exchange consists in the displacement of a given strand [(+)-strand] in a dsDNA, by another strand of the same polarity. Hence, the (−)-strand exchanges a (+)-strand for another (−)-strand. Ideally, assessing whether a protein already known to facilitate DNA renaturation is also able to catalyze strand exchange requires the availability of (i) preparations of dsDNA totally free of ssDNA and (ii) conditions for strand exchange that preclude DNA denaturation. Otherwise, as will become clear from our results, there will be no other choice but to try and establish the distinction between true strand exchange and...

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**Figure 1.** (A) Schematic map of murine p53 protein (390 aa) and mutant proteins 1–360 and 280–390. Identified on the map of the WT protein are the N-terminal transactivation and TBP binding domain (Transact.), hydrophobic region (Hydroph.), sequence-specific DNA binding domain, tetramerization region (Tetram.) and basic ss DNA binding domain (Basic). For a more detailed map see ref. 24. (B) Electrophoresis of purified WT and mutant proteins. A 1 µl sample from each purified protein was subjected to SDS–PAGE, and the gel was silver-stained. Lane M was run with a molecular weight marker (Pharmacia). Note that an added proline- and histidine-rich N-terminal tag (39,41) is responsible for each protein migrating slightly more slowly than expected from its calculated molecular weight.

**MATERIALS AND METHODS**

**Templates**

Oligonucleotides were synthesized with a Gene Assembler Plus synthesizer (Pharmacia-LKB). Two 30mer oligonucleotides (oligo 1 and oligo 2) and a 60mer (oligo 3) were used in most of this work. Oligo 1 (5'-ATTCTAAGCGACAGCGCTGC-3') was labelled at its 5'-end (renaturation, strand exchange and exonuclease assays) or at its 3'-end (exonuclease assay; see ref. 35). Oligo 2 (5'-GACACTGGTCACACTGCTTGCTGATTAAGAATTATAGTGGT-3') is complementary to oligo 1 and was used to prepare a 30 bp duplex (oligo 1+2) used in strand exchange experiments. Oligo 3 (5'-TTATGTTAGTTAGGACACTGTTGCTGACCTTGCTGCTGCTGTCGCTGATTAAGAATTATAGTGGT-3') the underlined sequence being that complementary to oligo 1) was used in both renaturation and strand exchange assays.

**Reference proteins**

RecA and SSB from *E.coli* were purchased from Pharmacia. All protein quantifications were carried out by the Bio-Rad protein assay, which relies on the method of Bradford (38).

**Expression vectors**

Recombinant *Autographa californica* baculoviruses expressing either the wild-type (WT) or the 280–390 mutant murine p53 protein (39) were generously provided by Dr P. Tegtmeyer. These vectors code for proteins with hexa-histidine tags at their N-terminus, allowing purification through a metal affinity column (see below).

**Cells and cell extracts**

*Spodoptera frugiperda* cells (SF9 strain) were grown as a suspension at 27°C, in Grace’s medium (40) supplemented with 10% fetal calf serum. They were infected at a density of 2 × 10^6 cells per ml with recombinant baculovirus (~1 p.f.u. cell), diluted to 3 × 10^5 cells per ml, further incubated at 27°C and harvested 3 days later. After centrifugation at 2000 r.p.m. in a Sorvall RC-3 centrifuge at 4°C for 20 min, the pellet from one liter of cell suspension was resuspended in 20 ml of lysis buffer (50 mM Tris–HCl pH 8.0; 150 mM NaCl; 1% NP-40; 10% glycerol; 1 mM phenylmethylsulfonyl fluoride; aprotinin and leupeptin, 50 µg/ml of each; 1 mM benzamidin; 5 mM β-mercaptoethanol) and left on ice for 30 min. After removal of cell debris (2000 r.p.m. for 15 min at 4°C in a lab top centrifuge), the nuclei were pelleted by centrifugation for 30 min at 20 000 r.p.m. and 4°C in a Sorvall SS34 rotor, and the supernatant (S100) was collected.

**SDS–PAGE**

Purified proteins were analyzed and quantified by SDS–PAGE (Fig. 1B) according to Laemmli (42) and silver stained as described by Wray et al. (43).
EDTA). In a control experiment, the ds material was eluted in TC buffer (10 mM Tris–HCl pH 7.5; 10 mM CaCl$_2$). In general, 0.1 ng of oligo 1+2 was mixed with 0.05 ng of oligo 3 (ratio of two duplexes 1+2 per ss oligo 3). Some experiments, however, were done at high DNA concentration and with a different ratio of ds to ss oligonucleotides, i.e., 6 ng of oligo 1+2 and 12 ng of oligo 3 (Fig. 3). Reactions were performed in 15 µl of p53 buffer (5 mM Tris–HCl pH 7.5; 10 mM KCl; 0.5 mM EDTA; 1.5 mM DTT; 1 mg/ml BSA; 3.7% glycerol; see ref. 31). Divalent cations were added to this buffer whenever appropriate (see Results). RecA was used as a control, and incubated with oligonucleotides in p53 buffer containing 4 mM ATP and an ATP-regenerating system [10 U/ml of creatine kinase (Boehringer) and 8 mM of phosphocreatine (Sigma)]. WT, mutant 1–360 and mutant 280–390 p53 proteins and SSB were tested in reaction mixtures including oligo 1+2 as well as oligo 3 [assays were carried out at protein concentrations, or protein–DNA mass ratios, that were comparable]. The protein was pre-incubated with oligo 3 for 10 min at 37°C, in p53 buffer with or without divalent cations, and the reaction started by adding oligo 1+2. The mixture was further incubated for 30 min at 37°C. SDS and EDTA were then added to a final concentration of 0.17% and 26 mM, respectively. The deproteinized material was subjected to electrophoresis through 10 or 15% polyacrylamide gels, which were dried and autoradiographed. WT and 280–390 mutant p53 proteins displayed essentially the same properties whether they had been purified in our laboratory or that of Dr P.Tegtmeyer; both preparations of 1–360 protein which we tested came directly from the latter laboratory.

**Renaturation assays**

Renaturation was performed as detailed for DNA strand exchange (see previous section), without preincubation however, and using only oligo 1 and oligo 3 (0.05 ng of each, thus with 2-fold molar excess of oligo 1) as substrates. Reactions were stopped and samples analyzed as stated in the previous section. Note that the amounts of oligos used in our assays were determined on the basis of results such as those displayed in Figure 4.

**Exonuclease assays**

The exonucleolytic activity of p53 was tested as already described (35) with either 3’- or 5’-labelled oligo 1 as substrate. Reaction products were analyzed by electrophoresis through 20% polyacrylamide gels containing 7 M urea.

**RESULTS**

**Three strand reactions**

The strand exchange assay which we used is illustrated in Figure 2A. Oligo 1+2, which consists of two paired oligos that are not only complementary but also of identical lengths (30 residues), was mixed with the larger oligo 3 (60 residues) which carries the whole of the sequence in oligo 2 (Fig. 2A, left); this mixture was incubated at 37°C with p53, RecA or SSB and, at the end of the reaction, deproteinization was carried out before electrophoresis and autoradiography (Materials and Methods). Since only oligo 1 (coloured in green in Fig. 2) had been radioactively labelled (see Materials and Methods), the extent of strand exchange could theoretically be appreciated by comparing the intensities of the only two expected radioactive species, M (oligo 1+2; Fig. 2A, left) and S (oligo 1+3; Fig. 2A, right); clearly, M would move ahead of S during electrophoresis, oligo 3 being double the length of oligo 2 (Materials and Methods). An example of how this assay worked at relatively high concentration of reagents (oligos and proteins, see Materials and Methods) is shown in Figure 3. The data illustrate quite clearly some requirements of the M to S conversion for each of the three proteins. First, SSB gave rise to a S band, but only in the absence of Mg$^{2+}$ (Fig. 3, lanes 3 and 4), a cation known to protect dsDNA from the denaturing activity of SSB (36). Secondly, p53 also gave rise to a S band (Fig. 4, lanes 5 and 6), even though neither the occurrence of this band in the absence of Mg$^{2+}$, nor its disappearance in the presence of Mg$^{2+}$, indicated for p53 a mode of action similar to that of SSB (see below). Finally, RecA also yielded a S band, which was particularly strong in the presence of both ATP and Mg$^{2+}$ (Fig. 3, lanes 7 and 8), as expected from the well-characterized strand exchange activity of this protein (5,9). Remarkably, the RecA-mediated reaction was the only one to result in more than half of oligo 1 being transferred from oligo 2 to oligo 3 (see S to M ratios.

![Figure 2. Strand exchange (A), denaturation/renaturation (B), and renaturation (C) involving oligonucleotides (oligos). The radioactive oligo (oligo 1, 30 nt) is shown in green and its exact complement (oligo 2, 30 nt) in red. Oligo 3 (60 nt) is shown in black, except for the portion of it that is identical to oligo 2 (shown in red). Since only oligo 1 (species F) is radioactively labelled, only two molecular species should be detectable (see Materials and Methods) in either the A (M and S) or the C (F and S) reaction. In contrast, denaturation/renaturation (B) should manifest itself by two species (M and F).](https://academic.oup.com/nar/article-abstract/25/20/4004/1023820)
When oligos are used at high concentration, SSB, p53 and RecA all seem to act as strand exchange proteins. Oligo 1+2 (6 ng) was mixed with a 2-fold molar excess of oligo 3 (12 ng) and incubated in p53 buffer, without (odd lanes) or with Mg\(^{2+}\), and either without protein (lanes 1 and 2), or with SSB (1200 ng, lanes 3 and 4), p53 (500 ng, lanes 5 and 6) or RecA (3100 ng, lanes 7 and 8). In the case of RecA, ATP and an ATP-regenerating system was added to both reactions (lanes 7 and 8). Note that only two species (M and S) are observed in lanes 3, 5 and 8, and only one species (M) in the other lanes, as expected if strand exchange (Fig. 2A) occurred with RecA in the presence of both Mg\(^{2+}\) and ATP (lane 8), and with either SSB or p53 in the absence of Mg\(^{2+}\) (lanes 3 and 5).

Reactions carried out with low concentrations of oligonucleotides

Surprising among the results shown above was the mimicking of strand exchange by SSB. Indeed, strand exchange activity has never been claimed for this extensively characterized protein (9). Possibly, in our assay, SSB was actually denaturing oligo 1+2 (Fig. 3, M band) in a reaction inhibited by Mg\(^{2+}\), and renaturation was occurring during or after deproteinization (see Materials and Methods). If such was actually the case, the concentration of oligonucleotides in our reaction mixtures was probably too high to allow an adequate characterization of a protein endowed with renaturing activity, such as p53.

We thus decided to look for conditions which would preclude spontaneous renaturation between the two oligos yielding band S in Figure 3, i.e., oligo 1 (hot) and oligo 3 (cold). Constant amounts of oligo 1 (0.05 ng) mixed with variable amounts (from 0.025 to 2.4 ng) of oligo 3 were incubated at 37 °C in either p53 buffer, or p53 buffer added with Mg\(^{2+}\), Ca\(^{2+}\) (not shown) or Na\(^{+}\), and the two expected radioactive species (F and S, Fig. 2C) were separated by electrophoresis (Fig. 4). Two observations were made in this experiment. First, renaturation and/or duplex stability was rather poor in p53 buffer (see strong F band across the gel in Fig. 4A), as compared with p53 buffer with added Mg\(^{2+}\) (Fig. 4B); secondly, a mixture containing as little as 0.05 ng of oligo 1 and 0.05 ng of oligo 3 (vertical arrows, Fig. 4) was not subject to spontaneous renaturation unless the p53 buffer was added with 10 mM Mg\(^{2+}\) (or Ca\(^{2+}\), see legend of Fig. 4). Thus, in order to avoid spontaneous renaturation in subsequent experiments conducted in the presence of proteins, we decided to use 0.1 ng of oligo 1+2 and 0.05 ng of oligo 3 in three strand reactions, and 0.05 ng of each oligo 1 and oligo 3 in two strand reactions (see below). In so doing, we were actually adjusting our conditions to those already used by others working with p53 (31, 34; see Materials and Methods).

With these lower concentrations of oligos, it now became clear that most of oligo 1+2 (species M) was denatured by SSB (Fig. 5A, lane 3), oligo 1 thus migrating mostly as species F (Fig. 2B, right), and not as a mixture of species S and M (Fig. 2B, right) as before (Fig. 3, lane 8). While suggesting efficient strand exchange, this result was not surprising; indeed, the sequence complementary to oligo 1 was twice as abundant as oligo 3 than as oligo 2 (see Materials and Methods, and legend of Fig. 3).

As to p53, it was now clear that it acted differently from either SSB or RecA. In amounts varying between 15 and 50 ng per reaction, p53 was quite effective in generating S material, but mostly, if not exclusively, at the expense of F material (Fig. 5A, lanes 5 and 6). Such F material was also detectable in the absence of protein (Fig. 5A, lane 1), unless Mg\(^{2+}\) cations were added to the buffer (Fig. 5A, lane 2). We tentatively concluded from these
observations that oligo 1+2 undergoes partial denaturation either during incubation in p53 buffer or before (see below), and that Mg
2+ cations or low amounts of p53 allow renaturation yielding the S species (1+3, Fig. 2B), p53 being more effective than Mg 2+
in stimulating such renaturation. When p53 was used in amounts in excess of 50 ng however, the F band became undetectable, as
in stimulating such renaturation. When p53 was used in amounts increasing amounts of p53. The occurrence of oligo 1 (component F) in reaction mixtures containing neither Mg 2+ nor p53 (Fig. 5A, lane 1) was not too surprising in view of the results illustrated in Figure 4. However, it was still unclear whether this material originated during isolation of oligo 1+2, or while the three strand reaction was being carried out. To answer this question, we ran some reactions with oligo 1+2 isolated in TC rather than TE buffer (Materials and Methods) prior to incubation in p53 buffer. Unlike TE buffer, TC buffer contains 10 mM CaCl 2 , which we expected to stabilize the ds structure of oligo 1+2. Such experiments indicated that this precautionary measure reduced but did not eliminate the occurrence of S material in p53 buffer (results not shown). Thus, incubation in p53 buffer necessarily leads to some denaturation of the 1+2 duplex.

As a control to the experiment in Figure 5A, we carried out a parallel experiment exploring renaturation between only two ss oligos, oligo 1 and oligo 3 (Fig. 2C). In this experiment, SSB had no detectable effect, as expected (Fig. 5B, lane 3). In contrast, p53 strongly stimulated the formation of S component (Fig. 5B, lanes 4–9), the optimal effect, however, requiring a larger amount of protein than in the three strand assay (Fig. 5A, lanes 4–8). Finally, 10 mM Mg 2+ cations turned out to be as effective as 150 ng of p53 in stimulating the formation of S component (Fig. 5B, lane 2). This had not been the case in the three strand assay (see above). The fact that p53 generates more S material than Mg 2+ in the three strand but not the two strand assay suggests that the two reagents have different reactivities with ss material and ds material (see Discussion).

Role of cations

In the next experiment, we decided to carry out three strand reactions (Fig. 6A) and two strand reactions (Fig. 6B) in the presence of a constant amount of p53 and of increasing concentrations of either of two divalent cations, Mg 2+ and Ca 2+. We already knew that, in the absence of protein, Mg 2+ and Ca 2+ exerted similar enhancing effects on renaturation and/or ds structure stability (Fig. 4). In the relatively straightforward two strand reaction (Fig. 6B), 10 mM of each cation was found to have about the same effect on renaturation as 100 ng of protein in the absence of cation (Fig. 6B, lanes 2, 3, 7 and 12), while no renaturation was detectable in the absence of p53 and either Mg 2+ or Ca 2+ (Fig. 6B, lanes 1 and 11). In addition we noted that, regardless of Ca 2+ concentration, the p53/Ca 2+ combination was no more effective than either of its components alone (Fig. 6B, lanes 7–10). Quite different results were obtained with the p53/Mg 2+ combination: the higher the Mg 2+ concentration, the weaker the S and F bands (Fig. 6B, lanes 4–6), and the greater the occurrence of smaller molecular weight radioactive material (Fig. 6B, lanes 4–6). The fact that the S band, while weaker, seemed to disappear even more rapidly than the F band was consistent with oligo 1 and oligo 3 (Fig. 2C) being separately subjected to degradation by a ss-specific exonuclease before they could renature (see below). The results of the three strand reaction (Fig. 6A) were consistent with this conclusion. At high concentrations of Mg 2+, the S band, but not the M band, virtually disappeared (Fig. 6A, lanes 5 and 6). Thus, the Mg 2+-dependent nucleolytic activity already detected in the two strand reaction appeared to have little effect on the ds template which already existed at the onset of the reaction (oligo 1+2, band M); in contrast, disappearance of the S band, and appearance of low molecular weight radioactive material (Fig. 6A, lanes 4, 5 and 6), again suggested exonucleolytic degradation of oligo 1 and oligo 3, that of oligo 3 having presumably started during reincubation, before renaturation or strand exchange could even take place. Once more (Fig. 5), whether in the absence or the presence of

Figure 5. SSB is a denaturing, and p53 a renaturing, protein. (A) Three strand assay (0.1 ng of oligo 1+2 and 0.05 ng of oligo 3). Note that three species are observed here (S, M and F) instead of two (S and M) in Figure 3. (B) Two strand assay (0.05 ng of each oligo 1 and oligo 3). Note that Mg 2+ is as effective as p53 in generating component S (this was not the case in A). The RecA reaction was carried out in the presence of ATP (4 mM) but in the absence of an ATP-regenerating system, i.e., under conditions previously found to be adequate for RecA-mediated DNA renaturation (44).
Figure 6. Action of divalent cations in three strand (A) and two strand (B) assays carried out in the presence of p53. The different oligos are used in the same amounts as in Figure 5. In this particular experiment, electrophoretic conditions were adjusted to allow demonstration of the effect of the Mg$^{2+}$/p53 combination on the molecular weight of the radioactive material. Note that while this combination has about the same effect on the S band in (A) and (B), the F band in (B) but not the M band in (A), is strongly reduced by the combined treatment (lanes 5 and 6).

Figure 7. Three strand (A) and two strand (B) reactions catalyzed by p53 mutants. The same amounts of oligos are used in the reactions as those indicated in Figure 5. Reaction mixtures including p53 uniformly contained 100 ng of protein (WT: 0.1 µM, 1–360: 0.11 µM, 280–390: 0.3 µM). Note that while mutant protein 1–360 is clearly defective in both reactions (A, lanes 5 and 6, and B, lanes 4 and 5), this is not the case for mutant protein 280–390 (A, lanes 7 and 8, and B, lanes 6 and 7).

divalent cation(s), p53 appeared somewhat more effective than either Mg$^{2+}$ or Ca$^{2+}$ alone in giving rise to an S band (Fig. 6A, lanes 2, 3, 7, 8, 9, 10 and 12). The main conclusion from this experiment was that divalent cations added little to the effect of p53 (WT at least, see below) in either of the two reactions carried out, except for the uncovering by Mg$^{2+}$ of a ss-specific nuclease activity which clearly was Mg$^{2+}$-concentration dependent.

p53 mutants

Deletion mutants of p53 were compared with the WT protein in three strand and two strand reactions, two different preparations of mutant proteins being tested simultaneously, preparations b and c for mutant protein 1–360, d and e for mutant protein 280–390 (Fig. 7). In the three strand assay, reactions run with Mg$^{2+}$, Ca$^{2+}$ or no divalent cation (no protein) were compared with reactions in which p53 was acting in the absence of such cations (Fig. 7A). It was clear from the data that the WT and the 280–390 mutant p53 proteins were at least as effective, if not more so, as divalent cations in prompting two effects: the disappearance of species F from, and the appearance of species S in, the reaction mixture (Fig. 7A, compare lanes 2 and 3 with lanes 4, 7 and 8). In contrast, mutant 1–360 appeared clearly less active than the other two proteins in both respects (Fig. 7A, lanes 5 and 6). Once again, the tight coupling of F species disappearance with S species appearance suggested that the two events were linked and probably reflected the renaturing activity of p53. In a second experiment, WT and mutant p53 proteins were compared for their ability to promote renaturation in the absence or presence of Mg$^{2+}$ cations (Fig. 7B). On this occasion, WT p53 proved clearly more active than Mg$^{2+}$ in stimulating renaturation, actually achieving complete conversion of the F species into S species (Fig. 7B, compare lanes 2 and 3). In the presence of WT p53 and Mg$^{2+}$, both species disappeared, again as expected from extensive exonuclease degradation of both oligos 1 and 3 (Fig. 7B, lane 8). Altogether different results were obtained with the mutant proteins. Renaturation was stronger with mutant 280–390 than with mutant 1–360 in the absence of Mg$^{2+}$, and either mutant protein performed as well or better in the
presence of cation as either Mg\(^{2+}\) or the protein alone (Fig. 7B, lanes 4–7 and 9–12). These data indicate that while WT p53 is the only one of the three proteins tested with readily detectable exonucleolytic activity, mutant protein 280–390 has renaturing activity which is virtually as strong as that of WT protein, as already observed by others (34; see also Fig. 1A).

Mutant protein 280–390 is reduced to the basic, non-specific DNA-binding domain of p53, and appears to lack exonuclease activity; yet, it is almost as active as WT p53 in our assays. This suggests that the effect produced by p53 in the three strand reaction is entirely attributable to the renaturing activity of this protein.

**Use of a modified three strand assay**

As indicated in Figure 2A, and also in Materials and Methods, the bulk of our strand exchange reactions were carried out with ds material consisting of two complementary oligos (1 and 2) of precisely equal lengths. We noticed that previous work on strand exchange by p53 had often resorted to complementary oligos differing in length by one, two or more residues (31,34). Conceivably, this could have markedly influenced the results. We thus decided to attempt strand exchange between oligo 3 and essentially double-stranded material, in which either oligo 1 or oligo 2 was shortened by two nucleotides, at either the 5′- or 3′-end (all four possible combinations were tried). The results obtained were no different from those obtained with fully double-stranded oligo 1+2 (not shown).

**Exonuclease activity of p53**

Mummenbrauer *et al.* (35) have described a 3′→5′, Mg\(^{2+}\)-dependent, single-strand-specific exonuclease associated with purified p53, as well as with some deletion mutants of this protein. We, of course, wondered whether this activity could explain the degradation of single-stranded oligonucleotides which we had found to take place during reaction with WT p53 in the presence of Mg\(^{2+}\) but not Ca\(^{2+}\) (Figs 6 and 7B). We thus tried to detect exonuclease activity in our preparations of proteins using precisely the conditions of Mummenbrauer *et al.* (35). Figure 8 shows the results obtained with WT p53 (designated Prep a in Fig. 7); our results could be superimposed on those of Mummenbrauer *et al.* (35) for the same protein. We also tested preparations of mutant proteins 1–360 and 280–390 for nuclease activity; while protein 1–360 possibly displayed a very weak activity, protein 280–390 appeared completely inactive (not shown). While the absence of a strong associated exonuclease activity with mutant protein 1–360 is at odds with the results of Mummenbrauer *et al.* (35), this data suggests that the exonuclease activity which we found associated with WT p53 is unlikely to be that of a contaminating nuclease. Actually, all three of the p53 proteins analyzed in Figure 7 had been purified by the same procedure, while the mutant proteins were used at somewhat higher molar concentrations (Fig. 7 legend) and yet only WT p53 appeared to possess exonuclease activity.

**DISCUSSION**

**Value of control proteins**

In this work, we resorted to an oligonucleotide assay (Fig. 2A) which, according to reports from two laboratories (31,34), allowed the demonstration of strand exchange by p53. In this context, both SSB and RecA permitted us to make useful comparisons. SSB, a protein which like p53 exists as a tetramer in solution, is known to have multiple binding modes to DNA; its effect on the structure of oligonucleotides of the size used in this work was expected to be strongly influenced by Mg\(^{2+}\) ions (45–47). Both SSB and RecA worked with oligonucleotides as we expected from the relevant literature, SSB being able to mediate denaturation (Fig. 5A) but not renaturation (Fig. 5B) in

**Figure 8.** Characterization of the exonuclease activity of WT p53. The conditions were those of Mummenbrauer *et al.* (35). 3′-labelling of oligo 1 (Materials and Methods) was performed with terminal transferase and [α-32P]dATP (lanes 1–10) and 5′-labelling with [α-32P]ATP and T4 polynucleotide kinase (lanes 11–15). 1–2 ng (10000–20000 c.p.m.) of labelled oligo 1 was incubated with (lanes 6–15) or without (lanes 1–5) 2 ng of WT p53 for the periods of time indicated above the different lanes. Reaction products were analyzed on a 20% polyacrylamide gel containing 7 M urea. Lanes 7–10 show the induction of ddAMP from the 5′-end of 3′-labelled oligo 1, and lanes 12–15 show the accumulation of a trinucleotide (tri), which is the end product of the reaction with the 5′-labelled oligo. Both data are in complete concordance with those of Mummenbrauer *et al.* (35) and suggest a 5′→3′ polarity for p53 exonuclease activity. Lane M, 5′-labelled oligonucleotide sizing markers from Pharmacia (lengths in nt are indicated on the left of the figure).
the absence of Mg$^{2+}$ cations; in contrast, RecA proved able to catalyze strand exchange (Figs 3 and 5A) but not denaturation (Fig. 5A) in p53 buffer added with Mg$^{2+}$ and ATP. It should be noted that our RecA-mediated reactions were all carried out in the absence of SSB. SSB is often added to the reaction mixture in which RecA-mediated strand exchange is carried out, even though the two proteins compete for binding to ssDNA, and strand exchange is known to occur in the absence of SSB (48,49). We omitted SSB when carrying out strand exchange with RecA because we found SSB to actually inhibit this reaction (D.Gendron, L.Delbecchi and P.Bourgaux, unpublished observations). Possibly, this effect was related to our use of oligonucleotides instead of DNA, which left SSB with little to achieve but impeding the binding of RecA (48,49). As to our use of a buffer containing ATP and Mg$^{2+}$ but no SSB to demonstrate RecA-mediated renaturation, it was consistent with earlier work on DNA renaturation (50,51), some of which indicated that SSB is inhibitory to this reaction (44). Actually, renaturation mediated by RecA in the presence of Mg$^{2+}$ (10 mM) and ATP was not obviously more extensive than that mediated by 10 mM Mg$^{2+}$ alone (Fig. 5B); this is perhaps not too surprising, since optimal DNA renaturation by RecA requires 30 mM Mg$^{2+}$ (44), a concentration of cations which deviates significantly from that used in this work.

Mechanism of action of p53: possible role of exonucleolytic activity in strand transfer

As alluded to in our Introduction, some proteins owe part of their strand exchange activity to being able to degrade rather than displace a given strand in a duplex DNA (1,23). Others (35) have described the association of exonucleolytic activity with p53 (WT or mutant) and our data (this work) are consistent with WT p53 being associated with such an activity. There is nothing in our data however that would support the notion that this activity would assist p53 in catalyzing strand exchange. In the three strand reaction (Fig. 2A), enhanced strand exchange should translate into a greater occurrence of S component. Yet, at any of the Mg$^{2+}$ concentrations tested, addition of this cation to the three strand reaction meant a decrease in the intensity of the S band (Fig. 6A). Also, mutant p53 protein with little or no exonucleolytic activity (Fig. 7B, lanes 11 and 12) proved almost as active as WT p53 in generating the S species during a three strand reaction (Fig. 7A, lanes 4, 7 and 8). Finally, the Mg$^{2+}$-dependent exonucleolytic activity associated with WT p53 appeared to preferentially degrade ss material (Fig. 6, compare lanes 4, 5 and 6 between A and B), and thus would be expected to impede strand exchange rather than facilitate it; indeed, digesting oligo 3, rather than oligo 2 in oligo 1+2, would be equivalent to degrading the invading strand rather than the strand that should be displaced or somehow disposed (23).

Mechanism of action of p53: role of divalent cations

Careful analysis of our data clearly indicates that p53 has renaturing activity which, in the two strand assay, results in the appearance of an S band. It would, however, be hard to argue that in the three strand assay, the same S band results from renaturation plus strand exchange, or strand exchange alone; actually, in this assay, the amount of S material generated in the presence of p53 appears to eventually match that of the F material detected in its absence (see, for instance, Fig. 5A). Yet, we were initially puzzled by the observation that p53 was consistently better than Mg$^{2+}$ in stimulating the generation of a S species, but only in the three strand reaction (Figs 5A, 6A and 7A). We believe that this apparent discrepancy reflects a critical difference between the mechanism of action of Mg$^{2+}$ and that of p53, which matches a critical difference between the two strand and the three strand assay. Presumably, Mg$^{2+}$ acts by favoring ds over ss structure, while p53 acts primarily by binding to, and favoring renaturation of, ss material (31,32,52,53). Thus, in the three strand reaction where p53 is preincubated with oligo 3 (see Materials and Methods), p53-carrying oligo 3 would be expected to react readily with any complementary oligo added to the reaction mixture, such as oligo 1 arising from oligo 1+2 via spontaneous denaturation. Such denaturation does not come as a surprise, in view of the apparent instability of the ds structure (Fig. 4A) in the p53 buffer employed here and elsewhere (31,34) to look for strand exchange between oligonucleotides. Thus, unlike Mg$^{2+}$, p53 will favor the renaturation of oligo 3 with oligo 1 over that of oligo 1 with oligo 2 (Fig. 2B). At high amounts of p53 however, oligo 3 might become saturated, such that excess protein would bind more and more to oligo 1 and/or oligo 2. Hence, regeneration of oligo 1+2 (species M) would compete with generation of oligo 1+3 (species S); this could be one reason for the amount of label in the S band leveling off beyond 50 ng of p53 per reaction (Fig. 5A, lanes 7 and 8). In the two strand assay in contrast, bound p53 will necessarily be shared between oligo 1 and oligo 3, a requirement which may explain why sizeable generation of S material requires 50 ng of protein, rather than 15 ng of protein in the three strand assay (Fig. 5, compare lanes 5 and 6 in A and B).

In conclusion, we find that in the three strand assay as carried out here, p53 does act as if promoting some displacement of oligo 2 by oligo 3. However, if our interpretation is correct, this displacement does not require more from p53 than the exercise of its renaturing activity.

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